THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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FIFTY-SECOND YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an

existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio*, who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. Inasmuch as the time and place of the Annual Meeting of Members is fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of said meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and

define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1939.

The accounts have been audited by Messrs. Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1939, the book value of the Endowment Fund in the hands of the Central Hanover Bank and Trust Company as Trustee, was

General Fund,	Securities (market $846,763.12)	$ 899,277.72
	Real Estate	24,860.00
	Cash	356.81
Library Fund,	Securities (market $148,086.70)	$ 161,767.17
	Real Estate	20,102.88
	Cash	12,538.80
		$1,118,903.38

The income collected from these Funds was as follows:

General Endowment	$35,471.86
Library	6,700.05
	$42,171.91

The income in arrears on these Funds at the end of the year was:

Arrears General Fund	$10,957.06
Arrears Library Fund	4,365.75
	$15,322.81
Arrears at the end of 1938	$16,968.86
an improvement of	$ 1,646.05

The dividends from the General Biological Supply House totalled $14,986.00, a slight increase over the year before.

Retirement Fund: A total of $4,060 was paid in pensions of which $726 was advanced from current funds. The book value of the securities and real estate in this Fund at the end of the year was .. $17,111.75
balance of cash 31.28

$17,143.03
of which 923.20

was due current funds for advances, leaving a net book value of $16,219.83

Plant Assets: The land (exclusive of the Gansett and Devil's Lane tracts) the buildings, equipment and library represent an investment of $1,812,054.80
less reserve for depreciation 539,462.84

or a net of $1,272,591.96

Income and Expenses: Income including a donation of stock by Dr. Frank R. Lillie valued at $10,500 exceeded expenses including depreciation of $25,013.41, by $11,176.50.

There was expended from current funds for plant account a net of $19,281.23 and in payment of mortgage and note indebtedness a total of $9,000.00.

At the end of the year the Laboratory owed $3,500 on a note, the last of the notes given for the purchase of the Bar Neck property, which

note has since been paid. It owed on Accounts Payable $3,993.22 against which it had accounts receivable of $9,585.34 and cash in bank of $14,790.37. The Laboratory now has no mortgage indebtedness.

In addition to the gift of $20,000 for Hurricane Damage from the Carnegie Corporation and a gift of 300 shares of Crane Company stock received from Dr. Frank R. Lillie, both received early in 1939 and referred to in my last report, the Laboratory received in 1939—

From anonymous donor toward purchase of Bay Shore Lot No. 6	$1,000.00	
From National Research Council toward purchase of X-Ray Tube	275.00	
From General Electric Company toward purchase of X-Ray Tube	150.00	
From General Electric Company, Vapor Lamp Division, Sodium Lamp	62.50	$1,487.50

and Dr. Oliver Strong made a contribution in the form of a sale of Bay Shore Lot No. 6 at a figure considerably below its market value.

Following is the balance sheet, the condensed statement of income and outgo, and the surplus account all as set out by the accountants.

EXHIBIT A

Marine Biological Laboratory Balance Sheet,

December 31, 1939

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee —Schedules I-a and I-b		$1,118,903.38	
Securities and Cash—Minor Funds—Schedule II		8,994.51	$1,127,897.89
Plant Assets:			
Land—Schedule IV	$ 111,425.38		
Buildings—Schedule IV	1,237,683.99		
Equipment—Schedule IV	174,335.50		
Library—Schedule IV	288,609.93	$1,812,054.80	
Less Reserve for Depreciation		539,462.84	
		$1,272,591.96	
Cash in Dormitory Building Fund		223.24	
Cash in Reserve Fund		24.65	$1,272,839.85

Brought forward			$2,400,737.74
Current Assets:			
Cash		$ 14,790.37	
Accounts and Notes-Receivable		9,585.34	
Inventories:			
Supply Department	$ 33,139.40		
Biological Bulletin	10,252.20	43,391.60	
Investments:			
Devil's Lane Property	$ 44,771.89		
Gansett Property	5,923.29		
Stock in General Biological Supply House, Inc.	12,700.00		
Other Investment Stocks	17,770.00		
Securities, Real Estate, and Cash —Retirement Fund List—Schedule V, viz.,			
Retirement Fund Portion	16,219.83		
Current Account Portion	923.20	98,308.21	
Prepaid Insurance		3,316.53	
Items in Suspense (Net)		210.95	$ 169,603.00
			$2,570,340.74
Liabilities			
Endowment Funds:			
Endowment Funds—Schedule III	$1,117,824.52		
Reserve for Amortization of Bond Premiums	1,078.86	$1,118,903.38	
Minor Funds—Schedule III		8,994.51	$1,127,897.89
Plant Liabilities and Funds:			
Notes—Payable a/c Bar Neck Property Purchase		$ 3,500.00	
Donations and Gifts—Schedule III		1,039,890.11	
Other Investments in Plant from Gifts and Current Funds		229,449.74	$1,272,839.85
Current Liabilities and Surplus:			
Accounts—Payable		$ 3,993.22	
Reserve for Additional Repairs and Replacements on account of Hurricane Water-Damage		2,403.49	
Current Surplus—Exhibit C		163,206.29	$ 169,603.00

EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE, YEAR ENDED DECEMBER 31, 1939

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 35,471.86		$ 35,471.86
Library Fund		6,700.05		6,700.05
Donations		10,500.00		10,500.00

Instruction	$ 9,131.39	9,995.00		863.61
Research	6,147.59	16,210.00		10,062.41
Evening Lectures	202.15		$ 202.15	
Biological Bulletin and Membership Dues	8,621.47	9,158.18		536.71
Supply Department—Schedule VI	37,412.21	35,239.33	2,172.88	
Mess—Schedule VII	24,913.02	26,040.73		1,127.71
Dormitories—Schedule VIII	22,978.26	12,966.90	10,011.36	
(Interest and Depreciation charged to above 3 departments—See Schedules VI, VII, and VIII)	*23,826.40*			23,826.40
Dividends, General Biological Supply House, Inc.		14,986.00		14,986.00
Dividends, Crane Company		300.00		300.00
Rents:				
Bar Neck Property		3,581.69		3,581.69
Bay Shore Property	82.61		82.61	
Howes Property	432.41	480.00		47.59
Janitor House	80.22	360.00		279.78
Newman Cottage	71.34		71.34	
Danchakoff Cottages	497.60	750.00		252.40
Sale of Library Duplicates		98.84		98.84
Apparatus Rental		1,030.76		1,030.76
Interest on Notes—Receivable		60.00		60.00
Sundry Income		40.50		40.50
Maintenance of Plant:				
Buildings and Grounds	23,410.66		23,410.66	
Chemical and Special Apparatus Expense	14,540.59		14,540.59	
Library Expense	7,806.06		7,806.06	
Truck Expense	568.90		568.90	
Workmen's Compensation Insurance	460.18		460.18	
Sundry Expense	18.90		18.90	
General Expenses:				
Administration Expense	12,342.08		12,342.08	
Endowment Fund Trustee and Safe-keeping	1,013.81		1,013.81	
Interest on Notes and Mortgage —Payable	537.50		537.50	
Bad Debts	337.38		337.38	
Reserve for Depreciation	25,013.41		25,013.41	
	$172,793.34	$183,969.84	$98,589.81	$109,766.31
Excess of Income over Expense carried to Current Surplus—Exhibit C	11,176.50		11,176.50	
	$183,969.84		$109,766.31	

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT, YEAR ENDED DECEMBER 31, 1939

Balance, January 1, 1939			$138,197.19
Add:			
Excess of Income over Expense for Year as shown in Exhibit B		$11,176.50	
Emergency Grant of Carnegie Corporation		20,000.00	
Donation on account of Hurricane Water Damage		10.00	
Excess of Marine Insurance over Estimated Loss		95.04	
Reserve for Depreciation Charged to Plant Funds		25,013.41	
Equipment Replacements Added to Plant		4,192.90	60,487.85
			$198,685.04
Deduct:			
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Land	$ 32.27		
Buildings	247.95		
Equipment	7,338.14		
Library	14,338.92	21,957.28	
Equipment Replacements		4,192.90	
		$26,150.18	
Less Received for Plant Assets Disposed of	2,676.05		
Loss on Equipment Charged off due to Hurricane Water-Damage	477.63	3,153.68	
		$22,996.50	
Payment on Plant Mortgage and Notes—Payable		$ 9,000.00	
Pensions Paid	4,060.00		
Less Retirement Fund Income	577.75	3,482.25	35,478.75
Balance, December 31, 1939—Exhibit A			$163,206.29

Respectfully submitted,

LAWRASON RIGGS, JR.,
Treasurer.

V. REPORT OF THE LIBRARIAN

Aside from the appropriation of $18,800 for the Library for 1939, which has not been changed for the past three years, the Executive Committee raised the yearly salary of one assistant by $50 and allowed $800 for extra service if necessary for the year 1939 only in order that the reprint collection might be completely checked. The annual sum

was apportioned at the beginning of the year as follows: books, $1000; serials, $6000; binding, $1500; express, $300; supplies, $500; salaries, $7200; back sets, $2350; total, $18,850. The appropriation was expended as follows: books, $236.88; current serials, $4749.84; binding, $2052.98; express, $122.18; supplies, $308.57; salaries, $7200; back sets, $4040.76; total, $18,711.21. Of the $800 allowed for extra help only $123.60 was spent and the remainder reverted to the Laboratory. During the year $98.84 was realized by the sale of duplicates and this sum will be retained by the Library for emergencies.

It is a source of satisfaction to be able to report (as of April, 1940) that practically all German journals and books have been received and paid for complete to the end of the year 1939.

The Woods Hole Oceanographic Institution appropriated $600 for the Library, the sum allowed by this Institution since 1936. This was expended to the amount of $525.91 and the account rendered to that Institution separately.

This year the Library received fewer current serials by 60 titles than in 1938 due chiefly to the Librarian's action in cutting off exchanges with the " Biological Bulletin " where the receipts from foreign countries had become completely inadequate or had actually ceased. This decline in number of exchanges will undoubtedly be enhanced during the next years. The current serials are now 1246 in number of which 430 are subscriptions, 390 (15 new) purchases of the Marine Biological Laboratory, 40 (1 new) of the Woods Hole Oceanographic Institution; 608 are exchanges, 536 (6 new) with the " Biological Bulletin "; and 72 (2 new) with the Woods Hole Oceanographic Institution publications; and 199 come as gifts to the former and 9 as gifts to the latter. The record shows 53 books purchased, 41 by the Marine Biological Laboratory and 12 by the Woods Hole Oceanographic Institution; 18 presented by the authors and 39 from publishers; and Dr. Knower presented to the Library a biography of William B. Wherry by M. Fischer. Completed back sets of serials number 34; as purchases of the Marine Biological Laboratory, 27; of the Woods Hole Oceanographic Institution, 1; by exchange with the " Biological Bulletin," 2; by exchange of duplicate material, 1; and by gift, 3; while purchases of the Marine Biological Laboratory partially complete back sets, 13; exchanges of the " Biological Bulletin," 3; exchanges of duplicate material, 5; and by gift, 3. Reprint additions number 3850; current for 1938, 1559; current for 1939, 632, and of previous dates, 1659. Of these 432 pamphlets came to the Woods Hole Oceanographic Institution. A summary of the current holdings for the Library is therefore 46,136 bound volumes and 112,777 reprints.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I present herewith a report of the fifty-second session of the Marine Biological Laboratory, for the year 1939.

1. *Recovery from Flood Damage.* During the past year the Laboratory has gradually recovered from the extensive damages caused by the hurricane and flood of September, 1938. The task of putting to rights the very great amount of apparatus, of replacing what could not be repaired, of rebuilding drawers and shelving, and of restoring the chemical room to working order, has necessarily been long in completion. But under the competent direction of our permanent staff it is now well along. We may take pride in the fact that during the summer of 1939 no one was seriously inconvenienced by lack of usual laboratory facilities or other services ordinarily provided. For this happy outcome we are indebted particularly to Dr. Pond and to Mr. Larkin who have given unsparingly of their time and skill.

2. *Plans for New Buildings.* The Committee on Policies and Future of the Laboratory, in its report published last year, stressed three main needs: (*a*) additional stack-room space for the Library; (*b*) the replacement of the present wooden buildings with a fire-proof structure of solid construction; and (*c*) increased facilities for the Supply Department. Plans for all three projects have been drawn up. The Library Committee has approved of the preliminary plans for an addition to the Library. This is to be placed at the north end of the Brick Building extending in the same direction, but set back to overlap only the width of the present stack room. By this arrangement the reading room will not be disturbed, while the new stacks will be assured plenty of light and ventilation. The additional space thus provided will undoubtedly be sufficient to house the increment of books, periodicals, and reprints for many years to come, and will be adequate for a large number of readers.

Tentative plans for a building to replace the old wooden buildings have been prepared. They provide increased space for class rooms, larger individual laboratories for instructors and other investigators, a lecture hall, conference rooms, and adequate space for various facilities required for experimental work. The building, which according to the present plans will extend from the site of the Lecture Hall eastward along Center Street, will house about the maximum number of investigators and students who now work in the present old laboratories. It is not the intention to provide for any substantial increase over that maximum.

The Committee on the Supply Department, in collaboration with Mr. Norris Jones, has prepared plans for utilizing more efficiently the buildings and space now occupied. They believe that the character of the present structures is, in general, consistent with the local surroundings and should be preserved. The Stone House will be little changed outwardly, but rebuilt within so as to provide space for the Museum now in preparation by Mr. Gray. A portion of the present Supply Department building will be transformed into laboratories adapted particularly for those who will carry on studies in the natural history of the Woods Hole region. To aid in this work, and to extend the area in which collections can be made, another boat, capable of fair speed, is needed.

In the preparation of all of these plans the Committees have had the whole-hearted coöperation of the architect, Mr. F. V. Bulfinch, who made numerous trips to Woods Hole to study the situation and to consult with the various members.

The Committee on Research and Equipment points out that new experimental procedures are constantly coming into use, and that it is impossible to foresee what may be needed in the future. For that reason they recommend that a special fund be set aside to provide for such contingencies. For the present, a number of highly desirable improvements should be made. Among these are additional de-humidifying rooms, constant temperature rooms, sterilizing and dark rooms, and shop space for investigators. Place for these facilities can be provided in the new building and in certain rooms of the Brick Building.

To finance these various projects, and some others which should be undertaken in the near future, and to add substantially to our endowment, approximately $1,000,000 will be needed. The Committee has sent to the Rockefeller Foundation, for their consideration, a statement of our needs, together with other pertinent information regarding the Laboratory.

3. *Acknowledgments.* The Laboratory is greatly indebted to the Rector of the Church of the Messiah at Woods Hole, Rev. Robert W. Nicholson, who has generously allowed us to use the greenhouse located in the rear of the Rectory. Its constant use during the summer by many investigators emphasizes the fact that we are in urgent need of a greenhouse of our own.

We are also indebted to the National Research Council whose grant made possible the purchase of an X-ray tube much needed in carrying on the work of this rapidly growing department of research.

Dr. Oliver Strong has made a substantial and very welcome contribution by selling to the Laboratory, at a price far below the market

value, his lot on the bathing beach. Funds for securing this property were provided by a generous friend of the Laboratory who wishes to remain anonymous. By this timely action we are now assured permanent possession of an adequate beach.

5. *Election of Trustees and Officers.* At the meeting of the Trustees in 1939, Prof. G. N. Calkins was elected Trustee Emeritus. The new Trustees elected at that meeting were Dr. J. H. Northrop and Dr. W. R. Taylor. As Secretary of the Trustees, to take the place of Prof. Calkins, who ably served in this capacity for many years, Dr. H. B. Goodrich was chosen.

6. *Memorials.* The Memorials of deceased Trustees, read at the Corporation meetings held in 1938 and 1939, are printed in this report.

7. *Scientific Record of Former Students.* A brief statement of the record of former students in our courses was presented at the 1939 meeting of the Trustees. The analysis of the data was subsequently completed and is included in this report. The conclusion may fairly be drawn that the courses of instruction are fulfilling their purpose in a most gratifying manner.

There are appended as parts of this report:

1. Memorials of deceased Trustees.
2. A report on the scientific record of students attending during the years 1918–1931.
3. The Staff, 1939.
4. Investigators and Students, 1939.
5. A tabular view of attendance, 1935–1939.
6. Subscribing and Coöperating Institutions, 1939.
7. Evening Lectures, 1939.
8. Shorter Scientific Papers, 1939.
9. The General Scientific Meeting, 1939.
10. Members of the Corporation, 1939.

Respectfully submitted,

CHARLES PACKARD,
Associate Director.

1. MEMORIALS OF DECEASED TRUSTEES

I. Memorial to Dr. E. P. Lyon

By W. E. Garrey

Elias Potter Lyon died on May 4, 1937 in his seventieth year. For forty years he was a staunch friend and supporter of the Marine Bio-

logical Laboratory. As a graduate student at the University of Chicago he conducted his early investigations in this laboratory. In 1898 he was appointed one of the teaching staff which organized the first course in physiology given at Woods Hole in 1899. As an active investigator he published many reports of work done here; these dealt with the animal tropisms, other phases of animal behavior and reactions to environment. He also contributed largely to studies on the early stages of embryonic development, particularly the rhythmic oxidation and chemical changes incident to egg cleavages. He possessed a remarkable ingenuity in devising simple methods and decisive experiments, probably the outgrowth of early training and teaching of physics. His colleagues well remember the hum and whir of centrifuges which followed his introduction of that method of studying organization in marine eggs.

Lyon taught physics at the Harvard School in Chicago, biology at Bradley Polytechnic Institute (Peoria, Illinois), and physiology successively at Rush Medical College, University of Chicago, St. Louis University Medical School and University of Minnesota.

As a teacher of physiology he impressed upon his students the importance of fundamental biology and he induced and aided many of them to study at the Marine Biological Laboratory. As Dean of the Medical Schools at St. Louis and Minnesota he exhibited a fine administrative sense and capability which was often demonstrated while he was a Trustee of this Laboratory from 1922 to 1930—a tenure he resigned only when his heavy administrative duties limited his Woods Hole contacts.

Interested ever in their welfare, Lyon was the true and sympathetic friend of youth; to his maturer intimates he was loved for his innate tolerance, kindliness and generosity—for his absolute lack of selfishness. These attributes coupled with his high scientific and scholarly attainments and his whole-hearted devotion to the Marine Biological Laboratory are the marks of his distinction among us. Therefore, be it

Resolved: That the Corporation of the Marine Biological Laboratory record in its minutes an acknowledgment of its indebtedness to Elias Potter Lyon for his many years of service to, and association with the Laboratory, and its sense of the deep loss sustained by his death. Be it further

Resolved: That a copy of these sentiments and resolutions be transmitted to his widow, Mrs. Nelle Eastman Lyon.

August 9, 1938

II. Minutes on the Death of Milton J. Greenman

By C. R. Stockard

The Corporation of the Marine Biological Laboratory record a sense of deep loss at the death of Milton J. Greenman which occurred on April 7th, 1937.

Doctor Greenman became a Trustee of this Laboratory in 1908 and served actively for twenty-eight years until he was made Trustee Emeritus in 1936. Throughout the time of his Trusteeship he was engaged as Director of the Wistar Institute and the relationship between that Institute and this Laboratory was most intimate and cordial. Through Doctor Greenman's good offices the Wistar Institute subscribed for five research rooms at this laboratory for a number of years during a period when its economic problems were very difficult. The generous arrangement provided that the occupants of the Wistar Institute rooms were to be appointed by the Director of this Laboratory and few supporting institutions have been so liberal in this regard.

At the Wistar Institute Doctor Greenman not only developed a scientific staff in a place which had previously been a museum, but he soon became convinced that the Institute could render great service in aiding the anatomical and biological publications of the country. Thirty years ago, in 1908, five morphological periodicals of national importance were brought together under the publishing management of the Institute. Before this time these journals had depended upon the efforts of private individuals and in 1903 one of the publications, the Journal of Morphology, of which an earlier Director of this laboratory had been co-founder, had ceased publication. Through the Wistar Institute, Doctor Greenman renewed publication of the Journal of Morphology. The ownership of the journals was for some time in the hands of the editorial boards but all are now the property of the Wistar Institute. Through the publication of these journals Doctor Greenman rendered broad service to the workers in this Laboratory and those in all biological laboratories of the country. Therefore, be it

Resolved: That Doctor Greenman's efforts in these several directions and his long interest and constant attendance at the annual meetings of this Laboratory are deeply appreciated by the Corporation and Trustees, and be it

Resolved: That a copy of these sentiments and resolutions be recorded in the Minutes of the Corporation, and that a copy be sent to the family of Dr. Greenman.

III. Memorial to Professor William Morton Wheeler

By G. H. Parker

William Morton Wheeler died suddenly in Cambridge, Massachusetts, April nineteenth, 1937, in his seventy-third year.

Professor Wheeler became a member of the Corporation of the Marine Biological Laboratory in 1890 and, with the exception of a few years, he remained so until his death. He worked in the Laboratory as early as 1891, only three years after its founding, and participated in the Nature Study Course given here in 1900. He was elected a Trustee of the Laboratory in 1919 and served in that capacity till 1935 when he was made a Trustee Emeritus.

Professor Wheeler's distinguished career as a naturalist began in his native town of Milwaukee where his interest in insects was fostered by Dr. and Mrs. G. W. Peckham. His acquaintance with the life of the sea was first made in this laboratory under Dr. Whitman and was further strengthened by work at the Zoölogical Station at Naples. In his early days, marine problems claimed a fair share of his attention, but as time went on he became more and more occupied with the study of insects and especially of ants. Of the three hundred and more contributions from his pen, the majority of them have to do with these animals. He was the recipient of many honors and distinctions, and in his death the world of science and the Marine Biological Laboratory in particular loses an eminent teacher and a renowned investigator.

Whereas: Professor Wheeler, whose high distinction in biological research is so widely known, was a Trustee of the Corporation of the Marine Biological Laboratory for some eighteen years, therefore, be it

Resolved: That the Trustees and Corporation of the Marine Biological Laboratory recognize with great sorrow that in the death of Professor Wheeler science has lost an unusual scholar and this Corporation a distinguished Trustee and a loyal advocate. And be it further

Resolved: That this resolution be adopted and recorded in the minutes of the Corporation and that a copy of it be sent to the family of Professor Wheeler.

IV. Memorial to Dr. H. H. Donaldson

By E. G. Conklin

In the death of Henry H. Donaldson on January 23, 1938, the Marine Biological Laboratory lost one of the original members of its Corporation, and a Trustee for more than 25 years. Dr. Donaldson became a member of the Corporation in 1888 and continued an annual

membership until his death. In the report of the Treasurer for 1888 he is listed as one of the original contributors to the support of the Laboratory. He was elected a Trustee in 1912 and re-elected in 1916, 1920, 1924; in 1929 he was elected Trustee Emeritus. With the exception of the sessions of 1920 and 1928, he occupied a research room at the Laboratory every summer after 1912. He served on various committees of the Trustees and his sound judgment and wise counsel were always available and of great value. Therefore, be it

Resolved: That the Corporation and Trustees hereby record their sorrow in the loss of a loyal and helpful member of our organization, a man of genuine scientific temper and of high ideals, and a personal friend of whom we have only happy and grateful memories. And be it further

Resolved: That these sentiments and resolutions be recorded in the minutes of the Corporation and that a copy be sent to Mrs. Donaldson.

IV. Memorial to Dr. E. B. Wilson

By Gary N. Calkins and T. H. Morgan

(Read by Dr. Calkins)

Edmund Beecher Wilson was born in Geneva, Illinois, in 1856, a son of Isaac G. Wilson and Caroline Clark Wilson. He attended Antioch College, the old University of Chicago, and the Sheffield Scientific School of Yale where he obtained the degree of Bachelor of Science in 1878; and three years later the degree of Ph.D. at Johns Hopkins University. He then studied in Cambridge, England, in Leipzig, and in Naples. During this period he made deep and lasting friendships with many of the leaders of European biology—Boveri, Butschli, Dohrn, Driesch, R. Hertwig and many others.

On returning to America, Wilson lectured for a year at Williams College and at the Institute of Technology. From 1885 to 1891 he was Professor of Biology at Bryn Mawr College. He was then called to Columbia University as Adjunct-Professor of Biology. Later he became Professor of Invertebrate Zoölogy, and then da Costa Professor of Zoölogy, holding the latter position until he was retired in 1928 with the title Professor Emeritus of Zoölogy in Residence in Columbia University. He died in New York City on March 3rd last.

Wilson's connection with the Marine Biological Laboratory began almost at its start in 1888, as he became a Trustee in 1889 and remained on the Board for the remainder of his life. Keenly interested in the welfare of the Laboratory, he took an active part in all the meetings of the Board.

Active and vigorous in youth and middle age, he was ready for athletic activity of all kinds, but bicycling, golf and sailing were the sports that he apparently liked best. His sensitive, artistic nature found ample expression in music and for many years not only did he patronize the musical centers of New York, but he was a devoted and excellent cello player, a member of a well-known New York string quartet, and a prominent member of the musical circles of that city.

He was a member of all of the leading learned societies of this country and Europe, and the recipient of innumerable honors here and abroad. But, significant as these are, he will be lovingly remembered by his many friends for the distinction of his mind and personality. His scientific keenness, judgment, and breadth of knowledge, were shown by the perfection of his lectures and papers.

He was the recognized leader in cytological research. His book on "The Cell in Development and Heredity" remains and will long remain a classic in this field. The American school of cellular research was, in large part, the outcome of his influence. His many devoted students and friends will remember him as their ideal of a scientific worker and charming companion.

V. Memorial to Dr. Charles R. Stockard

By Dayton J. Edwards

The Corporation of the Marine Biological Laboratory records with profound regret the death on April 7th, 1939 of Charles Rupert Stockard, a member of this scientific body since 1908 and an active participant in its affairs through a consecutive service of nineteen years on its board of Trustees.

Professor Stockard was born in Washington County, Mississippi, where his father was a practitioner of medicine. At an early age he was thus brought in touch with many of the every-day aspects of human biology and the sociological problems of the community. This heritage and these early experiences left imprints of deep significance in his life which in later years he frequently commented on; sometimes blending them into present-day situations and at other times contrasting them in effective and meaningful ways.

A preliminary college education in the Mississippi Agricultural and Mechanical College was completed in 1899 and soon thereafter he entered graduate work at Columbia University. In 1906 he became associated with the teaching staff of Cornell University Medical College. He was made Assistant Professor of Embryology in 1909 and two years later he was appointed Professor of Anatomy and Director of that De-

partment, a post he held for the remaining years of his life. Coincident with his academic assignments he maintained a vital interest in the Marine Biological Laboratory. This Institution held for him a peculiarly deep-seated significance and he was wont to recall his early associations here; also, the friendships with his old teachers, with his contemporaries, and with the group of younger biologists.

A bibliography covering a wide range of topics in the fields of cytology, embryology, genetics, endocrinology, medicine and education conveys at once an idea of Professor Stockard's versatility and symbolizes in a concrete way something of the genius, the originality, and the scholarliness of his mind. Approximately thirty-five of his earlier papers deal with problems on regeneration and the artificial production of structural anomalies in lower forms. His commanding knowledge of these problems was reflected prominently in much of his later work and teachings.

His engaging personality makes his passing a far greater loss than is indicated by his scientific achievement. A free and entertaining conversationalist, he was able to turn to the lighter sides of life with facility and enjoyment, and to witticisms of the most humorous nature. Endowed with a mind ready to challenge any height, and prompt to champion any cause he believed to be right, he possessed also a keen sense of scientific values, an incisive way of thinking and an unadorned form of expression.

In Charles Rupert Stockard, death has taken from this Corporation of the Marine Biological Laboratory one of the staunchest champions, one of its must faithful servants and helpful mentors.

Be it therefore Resolved: That the Members of the Marine Biological Laboratory hereby express their deep sense of the great loss which they have suffered through the sudden and untimely taking off of their fellow.

VI. Memorial to C. R. Crane

By Gary Calkins and T. H. Morgan

(Read by Dr. Morgan)

Charles R. Crane's beginning interest in this Laboratory can be placed approximately in 1901 when he was elected a member of the Corporation and a Trustee. But even before that date he had personal contacts with the work of the Laboratory which continued throughout his life. He became President of the Corporation in 1903, succeeding Professor H. F. Osborn, who resigned at that time. As soon as he became a Trustee he identified himself with the ideals and interests of the Laboratory.

His benefactions began in 1901, through personal gifts. When the contributions from the Carnegie Institution that extended from 1903 to 1905 came to an end, the Laboratory lived from hand to mouth, as it were, depending on its friends to cover its growing deficits. From 1910 to 1924 Mr. Crane, by most generous gifts, carried the burden of operating deficits almost alone. In addition he presented the first permanent building in 1913. In 1924 and 1925, in conjunction with the Rockefeller Foundation, Mr. John D. Rockefeller, Jr., and the Carnegie Corporation, Mr. Crane contributed largely to the erection and equipment of the second permanent building and to a sufficient endowment for future operations.

Quoting Dr. Lillie:

"The history of the institution would have been very different indeed if it had not been for Mr. Crane. His support gradually developed confidence in the soundness of the institution, even though managed by professors, and was a major factor in securing large gifts from the Rockefeller Foundation, Mr. John D. Rockefeller, Jr., the General Education Board and the Carnegie Corporation."

At the dedication of the Crane Laboratory in 1914 Mr. Crane said:

"I think we have come here particularly to celebrate the wonderful spirit that is back of the Woods Hole Biological Laboratory. It is very difficult to define that spirit, but I think we all know something of it and something is also known all through the scientific world. Without that spirit no amount of bricks and mortar and organization would be of any great service, but with that spirit the laboratory has been able to accomplish a very great deal with very simple means." (Director's Report for 1914, BIOLOGICAL BULLETIN, Vol. 28, No. 6, 1914, p. 345.)

Dr. Lillie commented recently: "This statement, characterized by brevity, appreciation of others, understanding of aims, and quiet humor gives admirably the spirit that animated all his acts for the Laboratory."

Let me quote, again, from Mr. Crane's address in 1925 on the occasion of the dedication of the second permanent laboratory:

"Even though not personally associated with the vital processes of the Laboratory, it has been the greatest possible privilege to play the part of a simple spectator in watching the growth of the wonderful spirit of coöperation in the work of biological research.

"Some years ago the then business manager of Rockefeller Institute for Medical Research invited me to spend the evening with him and try to help him understand the nature and conditions of the spirit of the Marine Biological Laboratory. 'For,' said he, 'We all recognize that the spirit is there. It is the rarest thing that we know of, and we have many discussions as to its nature and the conditions under which it has come forth.' He then asked me if I had any theory about it. I answered that the essential thing, as it

seemed to me, was that it was the purest expression of the highest form of democracy—a form of Soviet directed by the highest rather than the lowest motives. . . .

"We all know that this spirit which we are so much concerned about has long been domesticated in the old buildings across the street and among the older biologists. Although the street is a very narrow one, the mission of inviting the spirit of the laboratory into the new and more modern buildings and giving it a longer lease of its great power is mainly up to the younger biologists now coming along. Much power to them!" (From *Science*, Vol. 62, 1925, 271–272.)

When, in 1925, Mr. Crane resigned as President of the Trustees, he wrote:

"Twenty-two years have now elapsed since I became President of the Marine Biological Laboratory. I have enjoyed with you watching the growth of the Laboratory during that period. With the strong interest and support that is now assured, I feel that my own work has been completed and I hereby tender you my resignation which I ask you to accept. . . .

"The future progress and prosperity of the Laboratory will always be a matter of great interest to me, quite as much as if I continued to be your President."

Other gifts he has made from time to time, most of them of great value to the Laboratory, but the gift that the members of the Laboratory will cherish as being the greatest was his appreciation and understanding of the work that has been carried on in the Marine Biological Laboratory.

Mr. Crane died February 14, 1939.

VII. Memorial to Dr. J. P. McMurrich

By Frank R. Lillie

James Playfair McMurrich, Emeritus Professor of Anatomy in the University of Toronto, died February 9, 1939 in his seventy-ninth year. He was born and educated in Toronto and received his early training in zoölogy there; he obtained his Ph.D. degree in zoölogy at Johns Hopkins University in 1885. He received the honorary degree of LL.D. from the University of Michigan in 1912, from the University of Cincinnati in 1923 and from the University of Toronto in 1931. He held numerous offices in scientific societies, including the presidency of the American Association for the Advancement of Science in 1922.

His entire life was one of great scientific activity in research, teaching, and administration. He was primarily a zoölogist; although the greater part of his life was spent as Professor of Anatomy in a medical school, he never took a medical degree. He published 107 scientific papers from 1882 to 1932, was the author of two text books well-known in their time, "Invertebrate Morphology" (1894) and "Development

of the Human Body" (1903), and edited two treatises on human anatomy. His research interests were wide, ranging from coelenterates, mollusks, crustaceans and ascidians to various vertebrate groups; and in the last years of his life, the history of anatomy.

At the Marine Biological Laboratory we remember him best in his invertebrate days. He came to the Marine Biological Laboratory first in 1889 while serving as an associate of Professor Whitman in Clark University. In that year, and for two succeeding years, he was instructor in the course in Invertebrate Zoölogy. He delivered evening lectures on "The Phylogeny of the Actinozoa" (1889), "The Gastrea Theory and Its Successors" (1890) and on "The Significance of the Blastopore" (1891). He was a member of the Corporation from 1890 to the time of his death, and from 1892 to 1901 he was a member of the Board of Trustees. His withdrawal from active participation in the affairs of the Marine Biological Laboratory was a natural consequence of the change in direction of his scientific work to anatomy in relation to medicine.

It is many years since McMurrich was actively concerned in our affairs, though his interest was lifelong; but there are still some of us who remember him at Woods Hole as a quiet, courteous gentleman and scholar, loyal to the interests of the Laboratory, a good friend, fond of life and sports, and withal of exceptionally fine character and quality.

VIII. Memorial to Professor Edwin Linton

By G. H. Parker

Professor Edwin Linton, a member of the Corporation of the Marine Biological Laboratory since 1898, died in Philadelphia June 4, 1939, in his eighty-fifth year. He came to Woods Hole first in 1882 and worked in the Laboratory of the United States Fish Commission temporarily located on the Buoy Wharf at Little Harbor. At the time of his death he was the last survivor of that original band of investigators who first brought biology to this immediate shore. From these early times till his death Dr. Linton was an unremitting student of the parasites of fishes, a subject to which he made many valuable and original contributions. Although his researches were carried out in the Laboratory of the Bureau of Fisheries he always took a keen and active interest in the affairs of the Marine Biological Laboratory. He became a corporate member of this institution in the year when, by invitation, most of the workers at the Fisheries Laboratory joined this Corporation, whose annual meetings he regularly attended.

He was a familiar figure to all who came to the Woods Hole Laboratories. He took a lively interest not only in the general scientific

activities of the community, but also in its play. He was a skilled actor, the first President of the reorganized M. B. L. Club, and an enthusiastic member of the Choral Club. He and his wife established at Washington and Jefferson College the " Edwin S. Linton Memorial Endowment " in memory of their son who gave his life in France during the World War. The income from this endowment is paid to the Marine Biological Laboratory and has been the means of enabling not a few students to work here. This and other generous acts associated with Dr. Linton's name will long be remembered. Dr. Linton's sense of social obligation led him always to be a willing participant in any movement for the general good. In fact, it was through a step to protect others that he met with the accident that cost him his life. We mourn his loss as that of an earnest, scientific worker and of a generous and loyal associate. Therefore, be it

Resolved: That the Corporation of the Marine Biological Laboratory records in its minutes its sense of the great loss it has sustained in the death of Edwin Linton and be it further

Resolved: That a copy of these sentiments and resolutions be transmitted to Dr. Linton's family.

IX. Memorial to Dr. C. B. Bridges

By A. H. Sturtevant

Dr. Calvin Blackman Bridges died on December 27, 1938, just a few days before reaching the age of 50 years.

Dr. Bridges first came to the Marine Biological Laboratory in 1912, while he was still an undergraduate. For the next twenty years he regularly spent the summers at Woods Hole, where much of his work on the genetics and cytology of *Drosophila* was done.

Dr. Bridges had an unrivalled familiarity with this material, and one of his outstanding characteristics was his readiness to help other workers by supplying laboriously prepared material and by giving freely of his time in an advisory capacity. His scientific work was a conspicuous example of the unselfish coöperation that is one of the ideals of the Marine Biological Laboratory.

X. Memorial to W. O. Luscombe

By Caswell Grave and F. M. MacNaught

(Read by Dr. Grave)

For forty-three years Walter O. Luscombe has been an interested member of the Corporation of the Marine Biological Laboratory. He

joined in 1896 at a time when the Laboratory needed friends and encouragement; before its future had been assured by a large and devoted membership of biologists and by contributions of substantial buildings and endowment.

Mr. Luscombe sensed the worth of the struggling institution that was seeking to make its home in his community, and his request for membership in the Corporation is to be viewed as the action of a responsible citizen giving public expression to the interest and good will of the whole community.

He died in July in his 88th year.

We shall miss him from our Annual Meetings which he regularly attended and we find satisfaction in recording our appreciation of his kindly interest and good will.

2. THE SCIENTIFIC RECORD OF STUDENTS IN COURSES AT THE MARINE BIOLOGICAL LABORATORY

In the report of the Committee on the Policies and Future of the Marine Biological Laboratory the statement is made that "We have stood by the principle that it is the business of the Laboratory to help to produce investigators as well as investigation; and we believe that it can be shown that our courses of instruction have contributed in an important way to this purpose, and, moreover, that they have been an important factor in the improvement of biological instruction and research throughout the country." The record of the students who have attended these courses in past years fully justifies this belief. A very satisfactory proportion has continued in productive research in colleges and universities.

The first analysis of student records was made by Dr. G. A. Drew in 1923. His results, which cover the years 1908–1917 inclusive, are shown in the accompanying table. Those students whose names appeared in the current edition of American Men of Science were regarded as eminently successful teachers and investigators.

	Total	In A. M. S.
Invertebrate Zoölogy	350	12 per cent
Embryology	197	24 " "
Physiology	93	20 " "
Botany	111	26 " "
	751	18 " "

Out of a total of 751 registered during these years, 136 or 18 per cent

are thus cited. This record was considered excellent; but the figures for subsequent years are still more satisfactory.

The present analysis begins with 1918 and extends through 1931. A total of 1776 students were enrolled, of whom 56 were residents of foreign countries. Since the latter are not eligible for citation in American Men of Science, their records are not included here.

The second table shows the total number of men and women in each course, the number of these mentioned in American Men of Science (1938 edition) and the percentage of successful students, judged by this criterion. In the column labelled All Students, it is seen that out of 1720, 509 men and women, that is, 30 per cent have been included. The record of the men is uniformly much better than that of the women (50 per cent against 14 per cent). The low score of the latter is not a reflection of their ability. A large proportion marry and cannot continue in scientific work. Furthermore, there are not many opportunities for women to pursue independent research. In the column labelled "Corr." the figures are corrected to account for students who have taken more than one course. Except in the case of Protozoölogy, these corrections are not significant.

In the remaining sections of the table the students have been classified in three categories; (1) Undergraduates; that is, those who have not begun graduate work; (2) Graduate students; and (3) Instructors, including all teachers from instructors to those of professorial rank. The chief point of interest here is the fact that in each category, the proportion of men whose names appear in American Men of Science is about the same in all of the courses. Thus, among the undergraduates this is about 36 per cent for each course; among the graduates it is 67 per cent. The only exception in the latter group is in Botany. But in view of the small number of men (6) the low score is not significant. The same comment applies to the percentages of instructors.

The record for the women students in the different classes in each of the three categories is decidedly lower than that of the men. Taking into consideration the fact already mentioned that many have no opportunity for continuing their scientific work, the proportion cited in American Men of Science is good.

In general, therefore, it appears that of all students attending courses at Woods Hole, about one in three are mentioned in American Men of Science. Among the men alone, fully half attained this distinction, a remarkably good record. While it would be wrong to assume that such students have been successful because they attended the summer courses, yet it is true beyond all doubt that they received at Woods Hole a stimulus which was an important factor in directing their scientific career.

Record of Students in the M.B.L. courses during 1918–1931 inclusive

		All Students			Corr.	Under-graduates			Graduates			Instructors		
		Total	AMS	Per Cent		Total	AMS	Per Cent	Total	AMS	Per Cent	Total	AMS	Per Cent
Invert. Zoology	M	308	144	47.0	47.0	213	78	36.9	61	41	67.2	34	25	73.5
	W	441	46	10.5	10.6	378	37	10.0	45	6	13.3	18	33	16.7
	T	749	190	25.4	25.4	591	115	19.5	106	47	43.3	52	28	53.8
Embry-ology	M	174	80	46.0	49.0	113	39	35.1	27	18	66.7	34	23	67.7
	W	197	35	17.8	17.7	143	19	13.3	31	7	22.6	23	9	31.3
	T	371	115	31.0	33.6	256	58	22.7	58	25	43.1	57	32	56.1
Physi-ology	M	126	70	55.6	59.6	56	20	35.7	43	30	70.0	27	20	74.1
	W	107	25	23.4	23.5	60	10	16.8	26	8	30.8	21	7	33.3
	T	233	95	40.8	42.2	116	30	25.9	69	38	55.1	48	27	56.3
Proto-zool.	M	100	57	57.0	64.7	37	13	35.1	27	18	66.7	36	26	72.2
	W	90	15	16.7	22.2	48	5	10.4	23	6	26.1	19	4	21.1
	T	190	72	38.0	45.9	85	18	21.2	50	24	48.0	55	30	54.6
Botany	M	50	21	42.0	44.7	34	12	35.3	6	3	50.0	10	6	60.0
	W	127	16	12.6	12.7	76	4	5.3	19	4	21.1	32	8	25.0
	T	177	37	20.9	21.4	110	16	15.5	25	7	20.0	42	14	33.0
Totals	M	758	372	49.1	51.3	453	162	35.8	164	110	67.1	141	100	70.9
	W	962	137	14.2	14.3	705	75	10.6	144	31	21.5	113	31	27.4
	T	1720	509	29.6	30.7	1158	237	20.5	308	141	45.8	254	131	56.0

Note: 56 foreign students have been omitted since they are not eligible in AMS.

3. THE STAFF, 1939

Charles Packard, Associate Director, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
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Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, University of California.
Frank R. Lillie, Professor of Embryology, Emeritus, The University of Chicago.

C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy, Emeritus, Harvard University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

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P. S. Crowell, Jr., Instructor in Zoölogy, Miami University.
F. R. Kille, Assistant Professor of Biology, Swarthmore College.
A. M. Lucas, Associate Professor of Zoölogy, Iowa State College.
S. A. Matthews, Assistant Professor of Biology, Williams College.
J. S. Rankin, Jr., Instructor in Biology, Amherst College.
A. J. Waterman, Assistant Professor of Biology, Williams College.

Junior Instructors

W. E. Martin, Assistant Professor of Zoölogy, DePauw University.
N. T. Mattox, Instructor in Zoölogy, Miami University.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

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Virginia Dewey, Research Assistant, Brown University.
G. W. Kidder, Assistant Professor of Biology, Brown University.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

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W. W. Ballard, Assistant Professor of Biology and Anatomy, Dartmouth College.
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Viktor Hamburger, Assistant Professor of Zoölogy, Washington University.
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PHYSIOLOGY

I. Investigation

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Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
M. H. Jacobs, Professor of General Physiology, University of Pennsylvania.
Ralph S. Lillie, Professor of General Physiology, The University of Chicago.
Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

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Robert Chambers, Professor of Biology, New York University.
Kenneth C. Fisher, Assistant Professor of Experimental Biology, University of Toronto.
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C. Ladd Prosser, Assistant Professor of Physiology, Clark University.
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BOTANY

I. Investigation

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B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
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E. W. Sinnott, Professor of Botany, Barnard College.

II. Instruction

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G. W. Prescott, Associate Professor of Biology, Albion College.
B. F. D. Runk, Research Fellow, University of Virginia.

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G. Failla, X-ray Physicist.
Elbert P. Little, X-ray Technician.
J. D. Graham, Glassblower.
T. E. Larkin, Superintendent.
Lester F. Boss, Technician.
W. C. Hemenway, Carpenter.
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Deborah Lawrence, Secretary.
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Beginning Investigators, 1939

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URIE, JOHN C., Graduate Student, Villanova College.
VON DACH, HERMAN, Assistant, Zoölogy and Entomology, Ohio State University.
WHERRY, JOHN W., Graduate Assistant and Tutorial Fellow, Northwestern University.
WILBUR, KARL M., Research Fellow, University of Pennsylvania.
WILDE, CHARLES E., JR., Student, Dartmouth College.
WOOLLEY, JOHN P., Zoölogy Assistant, Columbia University.
ZWILLING, EDGAR, Teaching Assistant, Columbia University.

Research Assistants, 1939

ALLEY, ARMINE, Demonstrator, McGill University.
ARMSTRONG, CHARLES W. J., Demonstrator in Biology, University of Toronto.
BADGER, ELIZABETH A., Research Assistant, University of Cincinnati.
BAKER, LINVILLE A., Laboratory Assistant, Lilly Research Laboratories.
BAKER, RICHARD F., Research Assistant, Columbia University, College of Physicians and Surgeons.
BEAM, C. A., Student, Brown University.
BENDER, JOSEPH C., Laboratory Assistant, Swarthmore College.
BILKA, PAUL J., Trinity College.
BLOCH, ROBERT, Research Assistant, Columbia University.
BOWEN, WILLIAM J., Bruce Fellow, Johns Hopkins University.
BRISCOE, PRISCILLA M., Research Assistant, University of Pennsylvania.
BROWNELL, K. A., Research Assistant, Ohio State University.
BUTTS, C. EARL, University of Maryland.
CALABRISI, PAUL, Fellow in Anatomy, George Washington University.
CAMPBELL, JOHN B. S., Research Assistant, University of Pennsylvania.
CECIL, SAM, Assistant in Fluid Research, Biochemistry, Vanderbilt University, School of Medicine.
CHAMBERS, EDWARD L., Washington Square College, New York University.
COSTELLO, HELEN M., Fellow by Courtesy in Zoölogy, University of North Carolina.
CRAWFORD, JOHN D., Alumnus, Milton Academy.
CROWELL, VILLA BAILEY, Miami University.
CURTIS, HOWARD J., Rockefeller Fellow, Johns Hopkins University Medical School.
DEWEY, VIRGINIA C., Research Assistant, Brown University.
DOWDING, GRACE L., University of Maryland, School of Medicine.
DYTCHE, MARVON, Graduate Student, University of Pittsburgh.
DZIEMIAN, ARTHUR J., Graduate Student, Princeton University.

EDERSTROM, HELGE E., Assistant, Northwestern University.
EDMAN, MARJORIE, Research Assistant, University of Cincinnati.
ERLANGER, MARGARET, Instructor, West Virginia University.
FINKEL, ASHER J., Research Assistant, The University of Chicago.
FOSTER, RICHARD W., Alumnus, Milton Academy.
FRASER, DORIS A., University of Pennsylvania.
FURTH, OLGA B., Cornell University Medical College.
GAINES, ELIZABETH, Research Assistant, Amherst College.
GENTHER, THEODORE S., Assistant, University of Cincinnati.
GETTEMANS, JOHN F., Rockefeller Institute for Medical Research.
GIBERT, J. GORDON, University of Cincinnati.
GRAND, C. G., Research Associate, Washington Square College, New York University.
GRAVE, CASWELL, II, Assistant, Washington University.
GRINNELL, STUART W., Research Associate, Swarthmore College.
HENDRICKS, ELLIOTT M., Assistant, University of Cincinnati.
HÖBER, JOSEPHINE, University of Pennsylvania.
HOWE, JOHN H., Student, Brown University.
HUTCHENS, JOHN, Laboratory Assistant, Lilly Research Laboratories.
IDOINE, LEON S., Assistant in Biology, University of Toledo.
KALISS, NATHAN, Research Assistant, University of Pennsylvania.
KEEFE, EUGENE L., Research Assistant, Washington University.
KLEINHOLZ, L. H., Harvard University.
LANDIS, ROBERT E., Graduate Student in Physiology, University of Pittsburgh.
LOVE, GENEVIEVE, Brookville, Pennsylvania.
MAPP, FREDERICK E., Student, Atlanta University.
MARTIN, ROSEMARY D. C., Assistant, University of Toronto.
MAXFIELD, MARY E., Assistant Instructor, University of Pennsylvania.
MAXWELL, THOMAS, JR., Student, University of Pennsylvania, School of Medicine.
MAYO, MERCEDES, Graduate Assistant in Biology, Universidad de la Habana.
MEYERHOF, BETTINA, Student, Swarthmore College.
MILFORD, JOHN J., Graduate Assistant, New York University.
MUSSER, RUTH E., Student, Goucher College.
NAUMANN, RUDOLPH V., Fellow in Physiology, New York University, College of Medicine.
NETSKY, MARTIN, Research Assistant, University of Pennsylvania.
PAPALE, VICTORIA L., Graduate Assistant in Biology, Montclair State Teachers College.
PAPANDREA, D. A., Student, Albany Medical College.
PEMBERTON, FRANK A., JR., Harvard University.
PHELPS, LILLIAN A., Milbury, New Jersey.
POOL, JUDITH G. DE SOLA, Graduate Student, The University of Chicago.
POOL, NAOMI DE SOLA, Student, Goucher College.
RAMSDELL, PAULINE A., Student, Swarthmore College.
RICCA, R. A., Student, University of Pennsylvania, School of Medicine.
SAFFORD, VIRGINIA, Assistant, Swarthmore College.
SCHAEFFER, OLIVE K., Research Assistant, Biological Institute, Philadelphia.
SCUDAMORE, HAROLD H., Assistant, Northwestern University.
SNEDECOR, GEORGE W., Director Statistical Laboratory, Iowa State College.
SOMMERS, JOHN H., Minneapolis, Minnesota.
STONESTREET, GARTH A., Milton Academy.
THOMPSON, RAY K., Research Assistant, University of Maryland.
TROMBETTA, VIVIAN V., Assistant in Botany, Columbia University.
WEAVER, HARRY L., Research Assistant, Columbia University.
WIGHTMAN, JOHN C., Science Instructor, Darrow School, New Lebanon, New York.

ZIMMERMAN, ALICE C., Assistant, Brown University.
ZWILLING, RHEA LYON, University of Maryland, School of Medicine.

Students, 1939

BOTANY

BOOTH, BEATRICE L., Student, Smith College.
DENMAN, THOMAS H., Student Assistant, Drew University.
FAIRCHILD, ROBERT S., Graduate Fellow, Iowa State College.
HANSON, ANNE M., Hunter College.
MYERS, DORIS U., Student, Barnard College.
PAGE, ROBERT M., Harvard University.
REYNOLDS, DONALD M., Student, Harvard University.
THIVY, FRANCESCA, Lecturer in Botany, Women's Christian College, Madras, India.
WHITESIDE, JULIA, Student, Wellesley College.

EMBRYOLOGY

BARDEN, ROBERT B., Graduate Student, Teaching Assistant, Stanford University.
BARKLEY, MABEL A., Teacher of Biology, Washington Public Schools.
BARNES, MARTHA R., Assistant Instructor, University of Illinois.
BIRMINGHAM, JOHN R., St. Johns College.
BLAKE, WILLIAM D., Student, Dartmouth College.
BOLIEK, MILDRED I., Instructor in Zoölogy, Florida State College for Women.
BRANDAU, LOUISE B., Goucher College.
BRASTED, ADAIR M., Graduate Assistant, University of Rochester.
BUEKER, ELMER D., Student, Washington University.
CARRERAS, CARLOS J. G., Graduate Assistant, Havana University.
CARROLL, KENNETH M., Student, Franklin and Marshall College.
CASTLE, RUTH M., Assistant in Zoölogy, Vassar College.
CHAPMAN, S. STEPHEN, Student, Harvard University.
DELANNEY, LOUIS E., Teaching Assistant, Stanford University.
DUSTIN, JOHN K., Amherst College.
FERGUSON, FREDERICK P., Graduate Assistant, Wesleyan University.
FORSYTH, JOHN W., Princeton University.
GILLETTE, ROY J., Graduate Assistant, Washington University.
HAMILTON, HOWARD L., Graduate Teaching Assistant, University of Rochester.
LANE, JEAN, Goucher College.
METCALF, WILLIAM G., Oberlin College.
METZ, CHARLES B., Johns Hopkins University.
OLSON, J. BENNETT, Teaching Assistant, University of California, Los Angeles.
PAULS, FRANCES, Oberlin College.
REED, ELEANORE, Assistant Professor of Histology, University of Pittsburgh.
SANGSTER, WILLIAM, JR., University of Virginia.
SCRIMSHAW, NEVIN S., Graduate Assistant, Harvard University.
SHAVER, JOHN R., Harrison Fellow in Zoölogy, University of Pennsylvania.
SINGER, MARCUS, Teaching Fellow, Harvard University.
TRINKAUS, J. PHILIP, Undergraduate Assistant, Wesleyan University.
TROWBRIDGE, CAROLYN, State University of Iowa.
WATERMAN, FREDERICK A., Professor of Zoölogy, Ohio Wesleyan University.
WATTERSON, RAY L., Graduate Teaching Assistant, University of Rochester.
WELLINGTON, DOROTHY, Wheaton College.
WILDE, CHARLES E., JR., Student, Dartmouth College.
YAMPOLSKY, MORRIS, Student, College of the City of New York.

PHYSIOLOGY

ARMSTRONG, FLORENCE H., Student Assistant, Dalhousie University.
BAKEWELL, MILDRED P., Bryn Mawr College.
BELL, TAINE TEMPLE, University of California at Los Angeles.

DAVENPORT, DEMOREST, Instructor in Biology, Reed College.
FULTON, GEORGE P., Teaching Fellow, Boston University.
GOULDING, HELEN J., Toronto, Canada.
HASSETT, CHARLES, Student, Johns Hopkins University.
HEMING, W. EDWARD, Instructor, Cornell University.
JONES, RICHARD J., Student Assistant, University of Buffalo, School of Medicine.
McVAY, JEAN A., Graduate Assistant, Northwestern University.
MAXFIELD, MARY E., Assistant Instructor, University of Pennsylvania.
PLIMPTON, CALVIN H., Amherst College.
POOL, JUDITH G. DE SOLA, The University of Chicago.
SCHOEPFLE, GORDON M., Assistant in Physiology, Princeton University.
SCOTT, JOHN W., Fellow in Physiology, University of Toronto.
STIMSON, ALLAN B., Undergraduate Assistant, Wesleyan University.
TIMCOE, HELEN M., Teaching Assistant, Rutgers University.
TITUS, BARBARA J., Graduate Assistant, Mount Holyoke College.
TOWNSEND, VIRGINIA, Resident Fellow, Mount Holyoke College.
WHERRY, JOHN W., Tutorial Fellow and Graduate Assistant, Northwestern University.
WORKMAN, GRACE W., University of Toronto.

PROTOZOÖLOGY

BRATENAHL, CHARLES G., Student, Williams College.
CHANG, LING-CHAO, Columbia University.
COE, FREDERICK W., Student, Ohio Wesleyan University.
DEVNEY, DOROTHY C., Instructor in Zoölogy, College of Scholastica.
GROUPÉ, VINCENT, Student, Wesleyan University.
HENRY, RICHARD J., Chemistry Laboratory Assistant, Gettysburg College.
MAMLET, NATALIE J., Graduate Student, Columbia University.
NEVIN, FLOYD R., Instructor in Biology, Cornell University.
REINSTEIN, CECIL R., Research Assistant, Emory University.
WAKSMAN, BYRON H., Student, Swarthmore College.
WALTHER, JEANNE P., Wheaton College.
YOLLES, STANLEY, Brooklyn College.

INVERTEBRATE ZOOLOGY

ALLEN, BARBARA, Graduate Assistant, Mount Holyoke College.
BACON, ROBERT L., Student, Hamilton College.
BARTLETT, LAWRENCE M., Massachusetts State College.
BRADLEY, FREDERICK, Howard University.
BROWNE, SARAH D., Student, Pennsylvania College for Women.
BUEKER, ELMER D., Graduate Student, Washington University.
CAVENDER, JOHN C., Assistant in Biology, Brothers College of Drew University.
CHAMBERS, GLADYS M., Assistant Professor of Biology, Tougaloo College.
CHRISTIANSEN, GERTRUDE M., Student, Wilson College.
DAY, ELIZABETH, Student, Elmira College.
DOUGLAS, PETER L., Student, Harvard University.
DUBOIS, REBECKAH, Student, Vassar College.
EGAN, RICHARD W., Undergraduate Assistant in Biology, Canisius College.
EHRMANN, IRENE, Student, State Teachers College at Montclair.
FALES, CATHERINE H., Graduate Assistant, Mount Holyoke College.
FRANK, SYLVIA R., Graduate Fellow, Columbia University.
GRIMM, MADELON R., State Teachers College, Montclair.
HALL, EVELYN J., Mount Holyoke College.
HALL, MARY N., Student Assistant in Zoölogy, Connecticut College for Women.
HARRISON, ROBERT W., Oberlin College.
HEMPHILL, CATHERINE D., Bryn Mawr College.

IFFT, JOHN D., Assistant, Yale University.
JENKINS, LUCILLE O., Student, Middlebury College.
KIDDER, ANNE M. M., Student, Bryn Mawr College.
KINCAID, JAMES H., Biology Instructor, Birmingham Southern College.
LININGER, RICHARD E., Student, DePauw University.
LOWER, GEORGE G., Teacher of Biology and General Science, Westtown Friends School.
LUDWIG, FRANCIS W., University of Pennsylvania.
MCCALLA, FRANCES L., Instructor, Agnes Scott College.
MANDREY, JEANETTE L., Graduate Assistant, Wellesley College.
MARZULLI, FRANCIS N., Johns Hopkins University.
MCDONALD, MALCOLM E., Graduate Assistant in Zoölogy, State University of Iowa.
METZ, CHARLES B., Student, Johns Hopkins University.
MOLNAR, GEORGE W., Graduate Student, Yale University.
MORRIS, ANNE, Student, Elmira College.
OPTON, EDWARD M., Graduate Student, Yale University.
POWERS, EDWARD L., JR., Student Assistant, Johns Hopkins University.
RAMSDELL, PAULINE A., Student, Swarthmore College.
RAYBURN, RUTH, Student, Oberlin College.
ROBERTSON, G. GORDON, Graduate Student, Assistant, Yale University.
ROGICK, MARY D., Professor of Biology, College of New Rochelle.
ROLLER, KATHRYN L., Graduate Student, Rutgers University.
SAMORODIN, A. J., Graduate Student, University of Minnesota.
SCHNEIDERMAN, FRANCES H., Student, Hunter College.
SPENCE, FRANCES L., Student, Oberlin College.
SWEIBEL, VIVIAN, Hunter College.
TERRY, ROBERT L., Earlham College.
TONKS, ROBERT E., Chairman, Department of Science, St. Andrew's School.
VOTER, MURIEL A., Instructor, Wheaton College.
WALKER, THEODORE J., Museum Assistant, University of Oklahoma.
WILLIAMS, CARROLL M., Austin Teaching Fellow in Anatomy, Harvard University.
WILLIAMS, LUCY F., Radcliffe College.
WINBORN, MORRIS K., Assistant, Amherst College.
WINSTEN, ALWIN, 1823 Marmion Avenue, New York City, New York.
ZABELIN, B. ELIZABETH, Teacher of Biology, Chicago High School.

5. A TABULAR VIEW OF ATTENDANCE, 1935–1939

	1935	1936	1937	1938	1939
INVESTIGATORS—Total	315	359	391	380	352
Independent	208	226	256	246	213
Under Instruction	56	76	74	53	60
Research Assistants	51	57	61	81	79
STUDENTS—Total	130	138	133	132	133
Zoölogy	55	55	57	54	55
Protozoölogy	16	17	16	10	12
Embryology	33	34	35	34	36
Physiology	20	22	16	22	21
Botany	6	10	9	12	9
TOTAL ATTENDANCE	445	497	524	512	485
Less Persons Registered as Both Students and Investigators	16	24	13	16	14
	429	473	511	496	471

INSTITUTIONS REPRESENTED—Total	143	158	165	151	162
By Investigators	111	120	134	125	132
By Students	70	77	79	67	72
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	—	2	3	4	2
By Students	3	3	2	1	2
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	7	9	16	14	8
By Students	1	5	—	3	1

6. SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1939

Amherst College
Atlanta University
Belgian American Educational Foundation
Bowdoin College
Brothers College of Drew University
Brown University
Bryn Mawr College
California Institute of Technology
Canisius College
Carnegie Institute of Washington
College of Physicians and Surgeons
College of William and Mary
Columbia University
Connecticut College for Women
Cornell University Medical College
Dalhousie University
Dartmouth College
DePauw University
Duke University
Elmira College
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Hunter College
Industrial and Engineering Chemistry, American Chemical Society
Iowa State College
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly & Co.
Long Island University
McGill University
Massachusetts State College
Memorial Hospital, New York City
Morehouse College
Mount Holyoke College
Mundelein College
New York State Department of Health
New York University, Washington Square College
New York University
Northwestern University
Oberlin College
Pennsylvania College for Women
Princeton University
Purdue University
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
St. Francis Seminary
St. Johns College
Smith College
State University of Iowa
Syracuse University
Temple University
Tufts College
Union College
University of Buffalo School of Medicine
University of Chicago
University of Cincinnati
University of Illinois
University of Maryland Medical School
University of Missouri
University of Notre Dame
University of Pennsylvania
University of Pennsylvania School of Medicine
University of Pittsburgh
University of Rochester
University of Toledo
University of Virginia
Vanderbilt University Medical School
Vassar College
Villanova College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
Wheaton College
Williams College
Wilson College
Yale University

7. EVENING LECTURES, 1939

Friday, June 23
Mr. John S. Garth ..... "Animal Life in Equatorial Pacific."

Friday, June 30
Dr. Harold H. Plough ..... "The Influence of Temperature in Evolution as Shown by Genetic Studies on Drosophila."

Friday, July 7
Dr. Gerhard Fankhauser ..... "Polyploidy in Amphibians."

Friday, July 14
Dr. Rudolf Höber ..... "Cellular Activity Released by Organic Compounds in Surface Reactions."

Friday, July 21
Dr. T. M. Sonneborn ..... "Sexuality and Related Problems in Paramecium."

Friday, July 28
Dr. Viktor Hamburger ..... "Correlations between Nervous and Non-nervous Structure during Development."

Friday, August 4
Dr. G. K. Noble ..... "Neural Basis of Social Behavior in Vertebrates."

Friday, August 11
Dr. George Wald ..... "Vitamin A and Vision."

Thursday, August 17
Dr. J. D. Bernal ..... "The Biological Significance of Protein Structure."

Friday, August 18
Dr. Joseph Needham ..... "The Metabolism of the Gastrula, with Reference to the Amphibian Primary Organizer."

Monday, August 21
Dr. Robert Chambers ..... "Micromanipulative Studies."
Dr. C. C. Speidel ..... "Living Cells in Action."

Thursday, August 24
Dr. Franz Weidenreich ..... "Pithecanthropus and Sinanthropus, the Most Primitive Types of Fossil Man, and Their Relation to the Later Stages of Human Evolution."

Friday, August 25
Dr. E. Sinnott ..... "The Relation of Cells to Organs in Plant Development."

Friday, September 1
Mr. Duncan M. Hodgson ..... "Congo Color."

8. SHORTER SCIENTIFIC PAPERS, 1939

Wednesday, July 5

Dr. George W. Kidder ..... "The Effect of Biologically Conditioned Medium on the Growth of Colpidium campylum."

Dr. John Hutchens ..... "Respiration in Chilomonas paramecium."

Dr. Jay A. Smith ..... "Temperature and Starch and Fat in Chilomonas paramecium."

Tuesday, July 11

Dr. Floyd Moser ..... "The Differentiation of Isolated Rudiments of the Amblystoma punctatum Embryo."

Mr. Robert H. Silber ..... "The Production of Duplicatas Cruciata and Multiple Heads by Regeneration in Planaria."

Dr. L. G. Barth ..... "Neural Differentiation without Organizer."

Tuesday, July 18

Dr. Kenneth C. Fisher ..... "The Effect of Substrate Concentration on the Cyanide Sensitivity of the Oxygen Consumption of Yeast."

Mr. C. W. J. Armstrong ..... "A Comparison of Cyanide and Azide as Inhibitors of Cell Respiration."

Dr. Laurence Irving ..... "The Relation of Blood to Respiratory Ability of Fresh Water Fish."

Tuesday, July 25

Dr. David R. Goddard ..... "The Relation between Fermentation and Respiration in Higher Plants."

Dr. Claude E. ZoBell ..... "The Rôle of Bacteria in the Fouling of Submerged Surfaces."

Dr. E. W. Sinnott ..... "Cell Division and Differentiation in Living Plant Meristems."

Tuesday, August 1

Dr. Leonard I. Katzin ..... "The Ionic Permeability of Frog Skin as Determined with the Aid of Radioactive Indicators."

Dr. Kenneth Bailey ..... "Crystallization of Myogen from Skeletal Muscle."

Dr. Theodor von Brand ..... "Chemical and Histochemical Observations on Macracanthorhynchus hirudinaceus."

Mr. C. L. Claff and
Dr. G. W. Kidder ..... "pH Reactions during Feeding in the Ciliate Bresslaua."

Tuesday, August 8

Dr. J. D. Ferry ..... "The Dielectric Properties of Insulin Solutions."

Dr. J. A. Kitching ..... "The Effects of Lack of Oxygen and of Low Oxygen Tensions on the Activities of Some Protozoa."

Dr. Herbert Shapiro ..... "Nerve Asphyxiation and Aerobic Recovery in Relation to Temperature."

Dr. D. A. Marsland ..... "Effects of Hydrostatic Pressure upon Certain Cellular Processes."

Tuesday, August 15

Dr. Grace Townsend ..... "On the Nature of the Material from Fertilizable Nereis Eggs Inducing Spawning of the Male."

Dr. W. C. Young ..... "Ovum and Spermatozoön Age at the Time of Fertilization and the Course of Gestation and Development in the Guinea Pig."

Dr. Cornelius T. Kaylor ..... "Experiments on the Production of Haploid Salamanders."

Dr. A. B. Novikoff ..... "Regulation in Mosaic Eggs."

Tuesday, August 22

Dr. R. M. Cable and
Dr. A. V. Hunninen ..... "Studies on the Life History of Spelotrema Nicolli."

Dr. Rita Guttman ..... "Stabilizing Action of Alkaline Earths upon Crab Nerve Membranes, as Manifested in Resting Potential Measurements."

Mr. D. L. Harris ..... "An Experimental Study of the Pigment Granules of the Arbacia Egg."

Dr. L. V. Heilbrunn ..... "The Action of Calcium on Muscle Protoplasm."

9. THE GENERAL SCIENTIFIC MEETINGS, 1939

Tuesday, August 29

Dr. D. P. Costello and
Miss R. A. Young ..... "The Mechanism of Membrane Elevation in the Egg of Nereis."

Mr. Edgar Zwilling ..... "Determination and Induction of the Anuran Olfactory Organ."

Dr. Ethel Browne Harvey ..... "A Method of Determining the Sex of Arbacia, and a New Method of Producing Twins, Triplets and Quadruplets."

Dr. Ethel Browne Harvey ..... "An Artificial Nucleus in a Non-nucleate Half-egg."

Dr. G. H. Parker ..... "Color Responses of Catfishes with Single Eyes."

Dr. Grace Townsend ..... "A Vibration Sense in a Swarming Annelid."

Dr. D. H. Wenrich ..... "Food Habits of Endamoeba muris."

Dr. J. E. Kindred ..... "A Quantitative Study of the Hemapoietic Organs of Young Albino Rats."

Dr. William H. F. Addison ..... "On the Histology of the Mammalian Carotid Sinus."

Dr. Alexander Sandow ..... "On Clark's Theory of Muscular Contraction."

Dr. A. Navez and
Mr. John D. Crawford ..... "Conditions Governing the Frequency of Contraction of the Heart of Venus mercenaria."

Dr. F. J. M. Sichel ..... "The Refractory Period in the Non-conducted Response of Striated Muscle."

Dr. Alice M. Russell ..... "Pigment Inheritance in the Fundulus-Scomber Hybrid."

Miss Virginia Safford ..... "The Use of the Swimbladder by Fish in Respiratory Stress."

Dr. Herbert Shapiro ..... "Water Permeability of Chaetopterus Eggs."

Dr. M. H. Jacobs and
Dr. A. K. Parpart ..... "A Mechanism of Increased Cell Permeability Resembling Catalysis."

Dr. M. E. Krahl,
Miss A. K. Keltch and
Dr. G. H. A. Clowes ..... "Oxygen Consumption and Cell Division of Fertilized Arbacia Eggs in the Presence of Respiratory Inhibitors."

Mr. M. G. Netsky and
Dr. M. H. Jacobs ..... "Some Factors Affecting the Rate of Hemolysis of the Mammalian Erythrocyte by N-butyl Alcohol."

Mr. J. B. S. Campbell and
Dr. M. H. Jacobs ..... "Studies on the Permeability-decreasing Effect of Alcohols and Pharmacologically Related Compounds on the Human Erythrocyte."

Dr. Richard G. Abell ..... "Quantitative Studies of the Rate of Passage of Protein and Other Nitrogenous Substances through the Walls of Growing and of Differentiated Mammalian Blood Capillaries."

Wednesday, August 30

Dr. Eric G. Ball and
Miss Bettina Meyerhof ..... "The Occurrence of Cytochrome and Other Haemochromogens in Certain Marine Forms."

Mr. Carl C. Smith and
Mr. David Glick ..... "Some Observations on Cholinesterase in Invertebrates."

Dr. Kenneth Bailey ..... "Crystalline Myogen."

Dr. J. O. Hutchens and
Dr. M. E. Krahl ..... "Effect of Increased Intracellular pH on the Physiological Action of Substituted Phenols."

Dr. Albert E. Navez ..... "Fatty Acid Compounds in the Unfertilized Egg of Arbacia punctulata."

Dr. Aurin M. Chase ..... "Color Changes in Luciferin Solutions."

Mr. Fred W. Alsup ..... "Photodynamic Action in the Eggs of Nereis limbata."

Mr. Irving Cohen ..... "Cleavage Delay in Arbacia Punctulata Eggs Irradiated While Closely Packed in Capillary Tubes."

Dr. Rubert S. Anderson ..... "The X-Ray Effect on the Cleavage Time of Arbacia Eggs in the Absence of Oxygen."

Dr. P. S. Henshaw ..... "Fixation of X-Ray Effect by Fertilization in Arbacia Eggs."

Dr. William R. Duryee ..... "Does the Action of X-Rays on the Nucleus Depend upon the Cytoplasm?"

PAPERS READ BY TITLE

Mrs. R. K. Abramowitz and
Dr. A. A. Abramowitz ..... "Moulting and Viability after Removal of the Eyestalks in Uca pugilator."

Dr. A. A. Abramowitz ..... "A New Method for the Assay of Intermedin."

Miss Florence Armstrong,
Miss Mary Maxfield,
Dr. C. Ladd Prosser and
Mr. Gordon Schoepfle ..... "Analysis of the Electrical Discharge from the Cardiac Ganglion of Limulus."

Mr. Robert Ballentine ..... "The Intra-cellular Distribution of Reducing Systems in the Arbacia Egg."

Dr. H. W. Beams and
Dr. T. C. Evans ..... "Some Effects of Colchicine upon the First Division of the Eggs of Arbacia punctulata."

Dr. E. Frances Botsford ..... "Temporal Summation in Neuromuscular Responses of the Earthworm, Lumbricus terrestris."

Dr. F. A. Brown, Jr. ..... "The Source of Chromatophorotropic Hormones in Crustacean Eyestalks."

Dr. F. A. Brown, Jr. and
Mr. H. H. Scudamore ..... "Comparative Effects of Sinus Gland Extracts of Different Crustaceans on Two Chromatophore Types."

Dr. F. A. Brown, Jr. and
Mr. H. E. Ederstrom ..... "On the Control of the Dark Chromatophores of Crago Telson and Uropods."

Dr. John B. Buck ..... "Micromanipulation of Salivary Gland Chromosomes."

Dr. T. C. Evans and
Dr. H. W. Beams ..... "Effects of Roentgen Radiation on Certain Phenomena Related to Cleavage in Arbacia Eggs (Arbacia punctulata)."

Dr. John D. Ferry ..... "Chemical and Mechanical Properties of Two Animal Jellies."

Miss Judith E. Graham and
Dr. F. J. M. Sichel ..... "Response of Frog Striated Muscle to $CaCl_2$."

Dr. Charlotte Haywood ..... "The Permeability of the Toadfish Liver to Inulin."

Miss Josephine Hollingsworth . "Activation of Cumingia and Arbacia Eggs by Bivalent Cations."

Dr. Dwight L. Hopkins ..... "The Vacuole Systems of a Fresh Water Limacine Amoeba."

Dr. Cornelius T. Kaylor ..... "Cytological Studies on Androgenetic Embryos of Triturus viridescens Which Have Ceased Development."

Dr. Valy Menkin ..... "Effect of Leukotaxine on Cellular Permeability to Water."

Dr. Valy Menkin ..... "Effect of Leukotaxine on Cell Cleavage."

Dr. Floyd Moser and
Dr. J. A. Kitching ..... "Response of the Arbacia Egg Cortex to Chemical and Physical Agents in the Absence of Oxygen."

Dr. S. Milton Nabrit ..... "Further Studies on Regeneration in Fundulus Embryos."

DR. A. J. WATERMAN ..... "The Action of Certain Drugs on the Intact Heart of the Compound Ascidian, Perophora viridis."

DR. OPAL WOLF ..... "An Effect of the Injection of a Solution of Dihydroxyestrin into Castrated Female Frogs, Rana pipiens."

DEMONSTRATIONS

Wednesday, August 30

MISS BETTINA MEYERHOF AND
DR. E. G. BALL ..... "The Cytochrome Spectra in Squid Heart."

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SEROLOGICAL REACTIONS AND SPECIES SPECIFICITY OF SOME HELMINTHS

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Introduction

Concepts regarding classification and relationships of organisms have been based chiefly on adult and developmental morphology, but the value of particular structures in determining descent has been variously interpreted by different authors. Accordingly, opinions concerning the phylogenetic status of certain forms have differed. Furthermore, failure to reach agreement as to what constitutes a species has provoked much discussion and controversy. The earlier studies dealing with morphology and development have been supplemented in recent years by the use of serological reactions. Long employed in the diagnosis and control of certain diseases, these reactions have been utilized also to determine species specificity and interrelationships of many organisms.

Serological methods have been increasingly stressed in experimental work designed to clarify phylogenetic relationships of parasitic worms and to test taxonomic systems based on morphological characters. Consideration of the several types of serological reactions indicated clearly that the precipitin test is best suited for the determination of species specificity and interrelationships of organisms. This test lends itself admirably to qualitative, quantitative, and *objective* interpretations, which are necessary and fundamental for the evaluation of serological relationships.

Historical Review

The precipitin reaction was discovered by Kraus (1897). He noted that blood sera of goats which had been inoculated with sterile cholera, typhoid or plague culture filtrates caused precipitates when mixed with their respective bacterial filtrates. The reaction was specific since only the filtrate of the bacterium used for immunization would produce a precipitate when mixed with the antiserum.

From the early researches of Bordet (1899) and of Tchistovitch (1899) it became evident that foreign proteins other than bacterial

ones were antigenic and could provoke the formation of precipitins when injected into an animal.

The discovery by Uhlenhuth (1901) that the precipitin reaction was not strictly specific, since an antiserum against one protein may react with other closely related proteins as well as with the protein used in its production, is the basis for the application of serological reactions to certain biological problems. He discovered that these reactions are quantitatively, as well as qualitatively, specific, i.e., any antiserum will react more strongly with the antigenic substance used in its formation (homologous reaction) than with other antigenically different, though closely related, substances (heterologous reactions), the degree of relationship being indicated by the strength of the heterologous reactions as compared to the homologous one.

Ascoli (1902), Graham-Smith and Sanger (1903), and Fornet and Müller (1908, 1910) modified Kraus' flocculation technique by introducing the antiserum into the precipitin tube below the antigen; at the zone of junction of the two a layer or "ring" of precipitate formed and the end-point or titer could be determined with greater precision.

Nuttall's (1904) extensive and outstanding study of several thousand qualitative and quantitative precipitin reactions with the blood of more than nine hundred species of animals confirmed, in general, the classification and relationships of these animals based on comparative anatomy and embryology.

A distinct advance was made by the work of Boyden (1926) who, employing Ascoli's "ring" technique, determined the titers and obtained confirmation of his quantitative reactions by reciprocal tests.

A. Aqueous Extracts in Serological Reactions

1. Alcohol-ether-soluble substances not removed

Research employing aqueous, helminthic antigens from which lipids had not been removed by preliminary alcohol-ether extraction has yielded interesting and suggestive, but variable and inconsistent, results. By injection of rabbits with increasing doses of a saline extract of *Taenia saginata*, Langer (1905) obtained antisera which yielded precipitates with both homologous and heterologous antigens.

Schwartz (1920) noted positive precipitin reactions of varying intensities between materials of *Ascaris*, *Belascaris*, *Toxascaris* and *Ascaridia*, depending on the degree of relationship of these members of the family Ascaridae (Ascaroidea). However, he obtained no precipitates between *Ascaris* antisera and the antigens of two genera, *Dictyocaulus* and *Strongylus*, belonging to the superfamily Strongyloidea.

Hektoen (1926) concluded that the precipitin reaction yields species-specific reactions and affords interrelationships which parallel those deduced from morphological comparisons of the cestodes and nematodes studied. The dilution factor for the homologous tests was variable, ranging from 1 : 400 to 1 : 10,000, but several of the *heterologous* reaction titers fell *within* this range.

Canning's (1929) report on precipitin reactions, employing various tissues of *Ascaris lumbricoides* and related ascarids, clearly indicated a serological differentiation of tissues of a single species as well as a variation in the reactions of homologous tissues from different species. Anti-tissue sera, the antigens of which were derived from a single germ layer, gave the strongest reaction with their homologous test antigen.

Eisenbrandt (1936, 1938) emphasized the importance of knowing the concentration of nitrogen in the extract, from which the protein content may be determined, but stated that "it cannot be said that the proteins are the only factors involved in these tests." Reciprocal tests confirmed the original ones in but a single case. Of further interest are the high titers which were obtained upon the injection of only 0.0096 gram of nitrogen (60 mg. of protein), and the extensive range of homologous titers which varied from 1 : 2,048,000 for *Toxocara canis* to 1 : 2000 for *Ascaridia lineata*, a range which included many heterologous titers.

Nitrogen-containing substances are not completely active, nor are they the only factor to be considered in serological reactions. Boyd and Hooker (1934) stated that "one has no assurance that all the antigenic 'nitrogen' is immunologically active." Viktoroff, Guintze-Verner and Demidova (1936) concluded that the precipitin titer of serum gives a more exact measure of its value than the amount of nitrogen in the precipitate. Levine and Moody (1939) stated that "Variation between species in total nitrogen content . . . does not form a significant variable." Antigens of greater nitrogen content did not produce antisera of *consistently* higher titer than that elicited by antigens of lower nitrogen content.

In addition to non-specific precipitation noted in reactions between lipid-containing antigens and immune sera, Eisenbrandt (1938) observed that "Sometimes the presence of lipids resulted in flocculation (of the antigen) even though the extract was sterile." Furthermore, lipids may cause precipitation even in normal sera, as reported by Mackie and Anderson (1937).

Group reactions have been noted also in complement fixation tests when lipid-containing, aqueous extracts were used as antigens. Chung

and T'ung (1939) concluded, on the basis of serological investigations involving hydatid and cysticercus cyst fluids, that complement fixation and intradermal tests are immunological group reactions to somatic cestode infections rather than specific reactions for the species involved in any particular case. The group specificity was not confined to cestodes or even to helminths, because the serum of kala-azar patients gave positive complement fixation reactions with hydatid cyst fluid; according to the authors, this positive reaction may be related to the high globulin content of the blood of kala-azar patients.

2. Alcohol-ether-soluble substances removed

In contrast to the variable and inconsistent results afforded by the use of whole-worm antigens, antisera produced by the injection of lipid-free antigenic materials tend to maintain species specificity when employed in serological reactions. Bachman (1928) and Augustine and Theiler (1932), after a preliminary extraction with ether to remove the lipoidal materials, extracted powdered *Trichinella spiralis* larvae with physiological salt solution. Specific, positive precipitin reactions in antigen dilutions of 1 : 2000 were obtained with antisera of injected animals. The latter authors reported positive intradermal reactions which paralleled those from the precipitin tests when antigen dilutions of 1 : 10,000 were used. In later reports, Bachman and Rodriquez-Molina (1932), using *Ascaris lumbricoides* and *Necator americanus* materials, recorded group-specific intradermal tests, but dilutions of only 1 : 200 were employed.

Stumberg (1930) performed precipitin and complement fixation tests with extracts of numerous helminths. Slightly higher titers were attained with the sera of rabbits injected with adult than with larval antigens, but the stages of the life cycle could hardly be considered serologically distinct. The antibodies produced in immunized rabbits were species-specific in antigen dilutions of 1 : 4000 or over, group-specific in dilutions from 1 : 1000 to 1 : 4000, and non-specific in dilutions below 1 : 1000.

It may be concluded from these data that species-specific serological reactions are more likely to result with antisera produced by the injection of lipid-free antigens than with those evoked by lipid-containing antigens.

B. Alcoholic Extracts in Serological Reactions

Extraction with alcohol yields antigens which are fundamentally lipoidal in nature. Lipids are unable to incite antibody formation by themselves but serve as haptens, since they can react with antisera produced by injection of lipid-protein mixtures. Landsteiner's studies

led him to the conclusion (1928), fundamental for all precipitin work, that a two-fold system of specificity exists in animals, the specificity of proteins and that of haptens. In the case of the proteins, the serological and chemical properties, in general, parallel closely the zoölogical relationships. On the other hand, reactions involving haptens are less perfectly correlated with the systematic position of species tested, since haptens of closely related species may be very different chemically and those of distantly related species very similar, if not identical. Manwaring (1934) reported that proteins of two distantly related species may be so completely homologized by artificial conjugation with the same hapten as to be readily mistaken for protein from the same species. Thus, lipids, being haptogenic substances and not *species*-specific in their reactions, could overshadow the species specificity of proteins, since antisera against such conjugates apparently contain both anti-protein and anti-hapten factors.

Group specificity has been obtained in complement fixation reactions when alcoholic extracts of trematodes have been employed as antigens. Fairley (1919–1933) noted group reactions with cercarial antigens of several species of *Schistosoma* and concluded, on the basis of complement fixation tests with lipid-containing materials, that there is an antigenic complex common to mammalian schistosomes. In contradistinction to Fairley, Cawston (1921) and Höppli (1921) reported group reactions between *Schistosoma* and *Fasciola hepatica* materials. Employing alcoholic cercarial antigen of *Schistosomatium pathlocopticum*, Tanabe (1923) obtained positive results with the antiserum of rats infected with this parasite as well as with sera of others harboring *S. japonicum*. In contrast to these results, species specificity was noted by Andrews (1935) for *S. japonicum* in complement fixation tests, *but* antisera of individuals infected with other species of *Schistosoma* were not tested.

Kolmer, Trist and Heist (1916) concluded that the complement fixation reaction is inadequate for differentiation of related species of cestodes, since the reactions indicated close relationship between the tapeworms, *Taenia serrata* (= *T. pisiformis*) and *Dipylidium caninum*, which are quite distinct morphologically. According to the authors, lipoidal substances present in the extracts were capable of absorbing complement even with luetic antisera. Wharton (1930, 1931) stated that tapeworm lipids are not true antigens but under certain conditions can influence the character of the antigenic proteins. The reaction of *Cysticercus pisiformis* antigen was relatively *weaker* with antiserum of its adult form, *T. pisiformis*, than with *Moniezia* antiserum, which indicates that antisera to lipid-containing antigens yield group-specific

reactions. Group specificity was recorded by Sievers (1935) for the tapeworms, *Bothriocephalus latus*, *Taenia saginata* and *Echinococcus*, but species-specific antigen components were found by quantitative methods of testing.

In experiments with nematodes, Kolmer, Trist, and Heist (1916) observed cross reactions indicative of close serological relationship between materials of *Ascaris canis* (=*Toxocara canis*) and *Strongylus gigas* (=*Dioctophyme renale*), species which are very different morphologically. Group reactions in the nematode family Filariidae were obtained by Fairley (1931), who used *Dirofilaria immitis* antigen to diagnose *Wuchereria bancrofti*, *Loa loa*, and *Onchocerca volvulus* infections by complement fixation methods.

Thus, it may be concluded that, in general, antigens prepared by alcoholic extraction of materials, and therefore composed basically of lipids, yield *group*-specific reactions. This group specificity was especially apparent when complement fixation technique was employed, and Neveu-Lemaire (1936) concluded that complement fixation tests yield no better than group specificity.

C. Purified Polysaccharides in Serological Reactions

Until recently, protein has been regarded as necessary for antibody formation and hence immunization, but it has been found that the polysaccharides may induce the formation of and react with antibodies. Though Zozaya (1932) obtained agglutinating and precipitating sera by injecting rabbits and horses with various polysaccharides adsorbed on collodion particles, Landsteiner and Jacobs (1932) failed to confirm this work. In earlier experiments, polysaccharides failed to stimulate the production of antibodies, because they had been altered considerably in the process of purification. Avery and Goebel (1933) reported that mice, though not rabbits, could be immunized by injections of natural acetylated, Type I pneumococcal polysaccharides, and the resulting immunity was type-specific. Heidelberger, Kendall and Scherp (1936) produced immunization to pneumococci by injections of protein-free preparations of their natural polysaccharides. Raistrick and Topley (1934) obtained antisera containing agglutinins and precipitins by the injection of a product from *Bacterium aertrycke* (=*Salmonella aertrycke*) which contained no demonstrable protein.

Purified polysaccharides of helminths have recently been shown to be antigenic independent of any protein. Using *Taenia taeniaformis*, Campbell (1936*a*) found that a polysaccharide fraction conferred a considerable degree of protection against infection with the onchospheres, though the proteins were more efficacious. Later he (1936*b*) reported that protein and purified polysaccharide fractions of *Ascaris*

lumbricoides were immunologically distinct in precipitin tests. Purified polysaccharides prepared by Campbell (1937) from a number of helminths were tested with whole-worm antisera by the precipitin reaction. Polysaccharide fractions exhibited a much greater specificity than the complex solutions of whole worms. The use of whole-worm antigens of the closely related forms, *Ascaris lumbricoides* and *A. suum*, indicated their specific identity, whereas polysaccharide fractions, when employed as antigens, showed differences between the two. It is evident, then, that purified polysaccharides of helminths can, even in the absence of protein, stimulate the formation of antibodies.

Species Specificity

Serological reactions have been employed rather extensively in studies on helminths, but divergent results have been reported by the several investigators. Langer (1905), working with *Taenia saginata*, *T. solium*, *T. cucumerina* (=*Dipylidium caninum*), and *Ascaris lumbricoides* materials, injected aqueous extracts which contained 500 mg. of protein, and he recorded a titer of 1 : 15,000 for species specificity. Bachman (1928) obtained a species-specific titer of 1 : 2000 upon administration of 240 to 500 mg. of *Trichinella spiralis* antigen. Canning (1929) injected only 40 mg. of dried, powdered *A. lumbricoides* antigen suspended in saline solution, but homologous titers varied from 1 : 1600 to 1 : 4000 in tests performed with antisera withdrawn from the rabbits ten to fifteen days after the last injection. Stumberg (1930) recorded species-specific titers of 1 : 4000 or greater upon the injection of 100 mg. of powdered cestode, nematode and acanthocephalan antigens. Wolfe (1935), working with mammalian blood sera, obtained a titer of 1 : 12,800 or greater for species specificity, but he administered 1.6 grams to 2.75 grams of protein by the long method of injection or 0.16 grams to 0.275 grams by the short method. Eisenbrandt (1938) reported a variation in the species-specific titers from 1 : 2000 to 1 : 2,048,000 with antisera produced by injection of cestode, nematode and acanthocephalan materials in aqueous solutions containing 60 mg. of protein. In experiments reported by Wolfe (1939), homologous titers varied from 1 : 16,000 to 1 : 1,024,000 when antisera produced by injection of mammalian, avian or reptilian sera were employed in precipitin tests. These high-titered antisera resulted from the administration of only 0.001 gram of protein per kilogram of body weight.

Moulton (1923) reported that chemical constancy or maturity in vertebrates is not established in the early stages of development. Manwaring (1934) stated that "marked changes in fractional specificities are also demonstrable in different stages of the normal life cycle of all plant, animal and microbic species thus far adequately studied."

Accordingly, the purposes of the present investigation are to determine: (1) the minimum amount of antigen necessary to provide reliable and constant precipitin reactions; (2) species-specific titers, thus giving an objective method for determining whether a form under consideration is a new species or merely a strain, variety or race of an established species; (3) the time in the life cycle at which species specificity is established, with a view to using serological methods in identifying the various larval with the adult stages of a parasite and thus aid in working life cycles; (4) interrelationships of the several species of trematodes and cestodes which were used; and (5) the value of the reciprocal tests for confirmation of the original results.

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Materials and Methods

Materials for establishing the time at which zoölogical specificity is attained have been supplied by several stages in the life cycles of the trematodes, *Parorchis acanthus*, *Cryptocotyle lingua* and *Zygocotyle lunata*, and the cestode, *Taenia taeniaformis*. In addition to these species, *Plagitura parva*, *Siphodera vinaledwardsii*, *Dipylidium caninum*, *Tetrabothrius* sp., and *Moniezia expansa* were used to determine the dilution limits for species specificity.

Preparation of Antigen

To free them of debris and foreign proteins, the worms, when obtained, were washed through several changes of physiological saline solution, filtered sea water, or distilled water, depending on the source of the parasite. Following this preliminary treatment, they were washed several times in sterile media and finally, if necessary, quickly through sterile distilled water to remove excess salts. Cotton-plugged sterile Florence flasks (liter-size), containing 500 cc. of wash solution, were used, and transfers of the worms were made by means of sterile pipettes. The parasites were transferred a minimum of six times, allowing half an hour or more in each flask, thus reducing the bacterial contamination to a minimum. During this washing procedure, the solutions which were used to wash worms from warm-blooded hosts were kept in the incubator at 37° C. Subsequent manipulations were done carefully to avoid contamination.

The living worms were frozen rapidly, using dry ice, and kept at this low temperature in a vacuum desiccator (residual pressure of 0.001

mm. Hg) over anhydrous calcium chloride until thoroughly dried. Rapid freezing preserves the original structure of labile substances, and, when the material has been thoroughly desiccated, it can be extracted with ether or absolute alcohol without destruction or denaturization of the proteins. The high vacuum causes rapid dehydration, and a certain amount of autorefrigeration would occur as a consequence of the rapid evaporation. Furthermore, this procedure removes almost all oxygen which could act on the tissue proteins and other antigenic substances to alter their native structure. Desiccation in the frozen state results in dried material which has a spongy texture and such material is more readily pulverized and more soluble than the flaky substances which result from dehydration at room temperature and atmospheric pressure.

After twenty-four hours in the vacuum, the worms were triturated, using an agate mortar and pestle, and then returned to the desiccator. Complete desiccation was indicated when the material arrived at a constant weight.

To remove the lipids, Bloor's mixture, which consists of three parts absolute ether to one part absolute alcohol, was employed. Extraction was done in Erlenmeyer flasks placed in a mechanical shaker oscillating at the rate of 220 times per minute. One part by weight of powdered worm material was mixed with one hundred parts by volume of extractant, and repeated extractions were made until the subsequent weight of the dry residue remained constant, at which time the material was assumed to be lipid-free.

After weighing the lipid-free residue, it was extracted for twenty-four hours (under constant agitation) with either physiological salt solution adjusted to a pH of 7.2–7.4 or with buffered saline solution maintaining a pH of 7.3. The pH of the test antigen solution must be approximately 7.2–7.4 to obviate the possibility of pseudo-positive reactions with antiserum.

After aqueous extraction the material was filtered through a sterilized Jenkins' bacteriological filter, and the sterile filtrate was transferred to sterile vaccine bottles. The residue was thoroughly desiccated and weighed. The exact amount of material, probably mainly protein and carbohydrate, in solution was determined by calculating the difference in weight of residues before and after the aqueous extraction. The clear, sterile filtrate was kept in the refrigerator until needed.

Antibody Production

For injection purposes, healthy rabbits which had been kept under observation several weeks prior to experimentation were used. All

injections were made into the lateral ear veins, since it was found that higher-titered antisera were produced by the intravenous than by the intraperitoneal route. Most satisfactory procedure involved four intravenous injections, one every third day, with gradually increasing dosage and with a ten-to-twelve day interval after the last injection before blood was withdrawn. This short method of antibody production in rabbits has certain advantages, which include the greater specificity of the antisera produced and the decrease in time involved in obtaining an antiserum. Furthermore, smaller amounts of antigen will yield antisera high in specificity for homologous antigen, whereas by the long method, in which large amounts have been used, the specificity may be masked, because the titers of heterologous reactions increase greatly while the homologous reaction titer becomes only slightly higher.

In the series of four injections, a total of 40 mg. of dry weight, lipid-free antigen was injected. The 40 mg. of antigen was divided into doses of increasing amounts, i.e., approximately 4, 8, 12 and 16 mg.

Ten to twelve days after the last injection, as much as 20 cc. of blood was withdrawn from the heart by a large, sterile hypodermic syringe fitted with a 19-gauge needle; the blood was placed in sterile, cotton-plugged centrifuge tubes and allowed to clot at room temperatures. Having clotted, it was placed in the refrigerator for several hours. Subsequent to centrifugation of the clotted blood, the serum was drawn off and used immediately for tests or placed in sterile tubes.

Precipitin Test

The usual technique for making the precipitin test was employed; 0.3 cc. of antiserum in a precipitin tube was overlaid by 0.3 cc. of variously diluted test antigen. The precipitin tubes were 4.0 mm. in diameter and were graduated in millimeters. Dilutions of the antigen were made with sterile, buffered saline solution maintaining a pH of 7.3. To make some of the precipitin tests it was necessary to evaporate the test solutions to the desired antigenic concentrations. Evaporation was effected by placing the extract in sterile Petri or crystallizing dishes in a partial vacuum over anhydrous calcium chloride. In the homologous reactions, antigenic concentrations of 1 : 1000, 1 : 2000, 1 : 3000, 1 : 4000, 1 : 6000, 1 : 8000 and 1 : 10,000 were used, and dilutions from 1 : 10 to 1 : 2000 were employed to determine the titers of the heterologous tests. In a positive reaction, a ring or layer of white precipitate occurred at the interphase of the serum and antigen. The width of the ring was dependent primarily on the strength of the antigen solution employed in making any particular test, provided antisera

of equivalent strengths were used. However, the width and density of the layer of precipitate were dependent secondarily on the degree of mixture which resulted when the antigen was placed over the serum.

The titer of a reaction is the highest antigen dilution which gives a visible precipitate when tested with an antiserum, either homologous

TABLE I

Homologous Tests to Determine Titers
(Each test performed in duplicate and many in triplicate)

Species	Temp.	1 : 1000	1 : 2000	1 : 3000	1 : 4000	1 : 6000	1 : 8000	1: 10,000
Parorchis Adult	Rm.	0	2.0 mm.	1.0 mm.	0.5 mm.	±	−	0
	37°C.	0	2.0 mm.	1.0 mm.	1.0 mm.	+	−	−
Parorchis Rediae	Rm.	0	2.0 mm.	1.0 mm.	1.0 mm.	+	−	0
	37°C.	0	3.0 mm.	1.5 mm.	1.0 mm.	0.5 mm.	−	−
Parorchis Metacercariae	Rm.	0	2.0 mm.	0	1.5 mm.	+	−	0
	37°C.	0	3.0 mm.	1.5 mm.	0.5 mm.	+	−	−
* *Cryptocotyle* Adult	Rm.	0	2.5 mm.	1.0 mm.	+	−	−	0
	37°C.	0	2.5 mm.	2.0 mm.	0.5 mm.	−	−	−
Cryptocotyle Cercariae	Rm.	0	2.0 mm.	1.0 mm.	0.5 mm.	+	−	0
	37°C.	0	2.5 mm.	2.0 mm.	1.0 mm.	0.5 mm.	−	−
Zygocotyle Adult	Rm.	0	1.5 mm.	0	0.5 mm.	+	−	0
	37°C.	0	2.0 mm.	1.0 mm.	0.5 mm.	+	−	−
Zygocotyle Metacercariae	Rm.	0	2.0 mm.	0	0.5 mm.	+	−	0
	37°C.	0	2.0 mm.	0	1.0 mm.	0.5 mm.	−	−
Siphodera Adult	Rm.	1.5 mm.	1.5 mm.	+	+	−	−	0
	37°C.	2.0 mm.	1.5 mm.	0.5 mm.	+	−	−	−
Plagitura Adult	Rm.	3.0 mm.	2.0 mm.	1.0 mm.	+	±?	−	0
	37°C.	3.0 mm.	2.0 mm.	1.5 mm.	0.5 mm.	+	−	−
Monieza Adult	Rm.	3.5 mm.	2.5 mm.	1.5 mm.	0.5 mm.	+	−	0
	37°C.	3.5 mm.	3.0 mm.	1.0 mm.	0.5 mm.	0.5 mm.	+(1)–(3)	−
Dipylidium Adult	Rm.	2.0 mm.	1.0 mm.	0	0.5 mm.	−	−	0
	37°C.	3.0 mm.	2.5 mm.	1.0 mm.	1.0 mm.	0.5 mm.	−(? in 2)	−
Tetrabothrius Adult	Rm.	3.0 mm.	2.0 mm.	0	0.5 mm.	+	−	0
	37°C.	3.5 mm.	2.0 mm.	1.0 mm.	0.5 mm.	+	−	−
T. taeniaformis Adult	Rm.	2.0 mm.	1.0 mm.	1.0 mm.	0.5 mm.	+	−	0
	37°C.	0	2.0 mm.	1.0 mm.	0.5 mm.	++	−	−
T. taeniaformis Cysticerci	Rm.	2.0 mm.	1.0 mm.	1.0 mm.	0.5 mm.	+	−	0
	37°C.	2.0 mm.	1.5 mm.	1.5 mm.	1.0 mm.	0.5 mm.	−	−

* Only 30 mg. of antigen injected.

or heterologous, the amount of antigen being determined on the basis of dry weight of antigenic material *actually in solution.*

In all cases the readings and titers were taken at the end of one hour, at room temperature in one series and at 37° C. in the other. As reported by Boyden (1926) and Eisenbrandt (1938), a period longer than one hour is unsatisfactory because the layer becomes diffuse and begins to settle out, resulting in poor determination of the end-point or titer.

Control tests were made, using (1) normal sera and antigen solutions, (2) immune sera and the extracting salt solution, and (3) normal sera and the extractant. "Normal serum" indicates the serum of the same rabbit as that used for immunization, the blood having been taken by intracardial puncture shortly before the series of injections was started.

Observations and Results

Tables were prepared to present the results of the experiments in graphic form. Table I shows the data from homologous precipitin tests performed at room temperatures and at 37° C. The width of the rings of precipitate was used to determine the effects of temperature variations on the reactions, and the titers of homologous reactions defined the end-point of a species-specific test. In the higher dilutions of the antigen, accurate measurement of the width of the ring in a positive reaction could not be made, and such tests were recorded by means of the plus sign (+); negative reactions were indicated by minus signs (−) and tests which were not performed, by zeros (0). Especially in the higher dilutions, positive reactions are more definite at 37°, and one must conclude that it is advisable to employ a constant high temperature (37° C.) in determination of titers of serological reactions.

Table II presents the titers of both homologous and heterologous reactions between the various species of trematodes, and Table III, similar data on the cestodes which were used in the experiments. The number in parenthesis indicates the number of tests from which an average was taken to denote the titer; the variation in the titers of individual tests was never more than plus or minus one dilution from the average. No reactions occurred between materials of the trematodes and cestodes which were tested. The limited amount of trematode antigens did not permit a comparison between heterologous reactions at different temperatures; therefore the tests, in which trematode material was used, were performed at the constant temperature of 37° C. With few exceptions, the titer which could be taken to be species-specific is 1 : 6000, but, in order that every case be included, it is necessary to assign a value of 1 : 4000 to species specificity. It may be noted that the titers of reactions are nearly the same when antigens prepared from various stages in the life cycle of a single species are tested with antisera produced by the injection of a single antigenic substance, either larval or adult. In cases of difference, however, the higher titer obtains in reactions with an antiserum tested against the same antigen which had been used in its formation. However, the

TABLE II

Homologous Titers Denoting Species Specificity
Heterologous Titers Denoting Relationships
Tests performed at 37° C.
(Number in parenthesis indicates number of tests)

Antiserum \ Antigen	*Parorchis* Adult	*Parorchis* Rediae	*Parorchis* Metacercariae	*Cryptocotyle* Adult	*Cryptocotyle* Cercariae	*Siphodera* Adult	*Plagitura* Adult	*Zygocotyle* Adult	*Zygocotyle* Metacercariae
Parorchis Adult	1 : 6000 (7)	1 : 4000 (8)	1 : 4000 (6)	1 : 600 (6)	1 : 400 (6)	1 : 250 (3)	1 : 60 (3)	1 : 10 (4)	1 : 10 (4)
Parorchis Rediae	1 : 4000 (8)	1 : 6000 (6)	1 : 6000 (6)	1 : 400 (8)	1 : 600 (10)	1 : 350 (3)	1 : 80 (3)	0 (10)	? (6) 1 :10(4)
Parorchis Metacercariae	1 : 4000 (8)	1 : 6000 (7)	1 : 6000 (4)	1 : 400 (7)	1 : 600 (6)	1 : 300 (3)	1 : 60 (3)	1 : 10 (3)	1 : 10 (5)
* *Cryptocotyle* Adult	1 : 400 (4)	1 : 400 (7)	1 : 400 (5)	1 : 4000 (6)	1 : 4000 (8)	1 : 1000 (7)	1 : 100 (3)	0	0
Cryptocotyle Cercariae	1 : 600 (5)	1 : 600 (7)	1 : 600 (6)	1 : 6000 (5)	1 : 6000 (6)	1 : 1200 (8)	1 : 150 (3)	0	0
Siphodera Adult	1 : 275 (7)	1 : 300 (9)	1 : 300 (7)	1 : 1200 (7)	1 : 1200 (4)	1 : 4000 (4)	1 : 30 (6)	0	0
Plagitura Adult	1 : 80 (4)	1 : 60 (4)	1 : 60 (3)	1 : 150 (6)	1 : 200 (6)	1 : 50 (7)	1 : 6000 (5)	0	0
Zygocotyle Adult	1 : 10 (4)	1 : 20 (6)	1 : 10 (5)	1 : 40 (3)	1 : 50 (3)	0	0	1 : 6000 (7)	1 : 4000 (7)
Zygocotyle Metacercariae	1 : 10 (5)	1:10(2) ?(3)	1 : 20 (6)	1 : 40 (3)	1 : 50 (4)	0	0	1 : 6000 (6)	1 : 6000 (6)

* Only 30 mg. of antigen injected.

TABLE III

Homologous Titers Denoting Species Specificity
Heterologous Titers Denoting Relationships
(Number in parenthesis indicates number of tests)

Antiserum \ Antigen	Temp.	*Moniezia* Adult	*Dipylidium* Adult	*T. taeniaformis* Adult	*T. taeniaformis* Cysticerci	*Tetrabothrius* Adult
Moniezia Adult	Rm.	1 :6000(5)	1 :400(6)	1 : 50(5)	1 : 50(5)	0
	37°C.	1 :6000(4)	1 :600(8)	0	1 : 70(4)	0
Dipylidium Adult	Rm.	1 :400(6)	1 :4000(4)	1 : 100(8)	1 : 100(7)	0
	37°C.	1 :400(6)	1 :6000(4)	1 : 150(7)	1 : 100(7)	0
T. taeniaformis Adult	Rm.	1 :50(7)	1 :100(4)	1 : 6000(6)	1 : 4000(6)	0
	37°C.	1 :50(7)	1 :100(4)	1 : 6000(4)	1 : 6000(4)	0
T. taeniaformis Cysticerci	Rm.	1 :60(5)	1 :100(5)	1 : 5000(5)	1 : 6000(5)	0
	37°C.	1 :70(5)	1 :100(5)	1 : 6000(4)	1 : 6000(5)	0
Tetrabothrius Adult	Rm.	0	0	0	0	1 : 6000(5)
	37°C.	0	0	0	0	1 : 6000(5)

difference was never greater than one dilution tube, which ordinarily is considered within the limit of experimental error.

Table IV was prepared from the data given in Tables II and III

TABLE IV

Relationship Expressed as Per Cent Values of Homologous Titers

Antiserum \ Antigen	Homologous Titer	*Parorchis*	*Cryptocotyle*	*Siphodera*	*Plagiura*	*Zygocotyle*	*Moniezia*	*Dipylidium*	*Taenia taeniaformis*	*Tetrabothrius*
TREMATODA										
Echinostomatidae										
Parorchis	1 : 4000	100	12.5	7.5	1.67	0.25	—	—	0	—
	1 : 6000		8.33	5.0	1.12	0.17				
Heterophyidae										
Cryptocotyle	1 : 4000	12.5	100*	27.5	3.13	0	—	—	0	—
	1 : 6000	8.33		18.3	2.08					
Siphodera	1 : 4000	7.3	30.0	100*	0.75	0	0	—	—	—
	1 : 6000	4.87	20.0		0.5					
Plagiorchidae										
Plagitura	1 : 4000	1.67	4.38	1.25	100	0	0	—	0	—
	1 : 6000	1.12	2.92	0.83						
Paramphistomidae										
Zygocotyle	1 : 4000	0.33	1.12	0	0	100	—	—	—	0
	1 : 6000	0.22	0.75							
CESTODA										
Moniezia	1 : 4000	—	—	0	—	—	100	12.5	1.5	0
	1 : 6000							8.33	1.0	
Dipylidium	1 : 4000	—	—	—	—	—	10.0	100	2.8	0
	1 : 6000						6.6		1.87	
Taenia taeniaformis	1 : 4000	0	0	—	0	—	1.25	2.5	100	0
	1 : 6000						0.83	1.67		
Tetrabothrius	1 : 4000	—	—	—	0	0	0	0	0	100
	1 : 6000									

* Relationship not 100 per cent when 1 : 6000 is considered homologous titer.

and presents the percentage of relationship of the various species based on homologous titers of 1 : 4000 and 1 : 6000, which were considered to be 100 per cent. Since the titers of reactions between various stages

in the life cycle of a species were nearly the same, the percentage values in Table IV are the result of a consideration of all stages in the life cycle of the species under examination in determining the percentage relationship of that species with another.

Precipitin reactions of high titer were secured by injection of as little as 10 or 20 mg. of antigenic material, but the results were not consistent, since corresponding titers did not result in every case. Injection of 40 mg. of antigen apparently is necessary to obtain reliable results, though further work may reveal methods for securing consistent titers upon administration of much less antigenic material.

Noteworthy in the present experiments are the confirmatory results yielded by reciprocal tests, which would seem to be necessary if serological reactions are to be significant in determination or confirmation of phylogenetic relationships.

In Table V the alcohol-ether-soluble contents of the helminths have been tabulated in percentages of the total dry weights. The amounts of these materials varied from 2.7 per cent in cysticerci of *Taenia taeniaformis* to 41.3 per cent in adults of *Tetrabothrius*. That the lipid content of a single species is not constant may be concluded from the different values obtained in two different extractions of *Parorchis acanthus* or of *Moniezia expansa*.

TABLE V

Alcohol-ether-soluble Substances of Helminths
(In per cent values of total dry weights)

Species		
Parorchis acanthus (adult)	(*a*)	28.62
	(*b*)	32.82
P. acanthus (rediae)		25.30
P. acanthus (metacercariae)		14.88
Cryptocotyle lingua (adult)		29.76
C. lingua (cercariae)		20.35
Siphodera vinaledwardsii (adult)		25.00
Plagitura parva (adult)		4.95
Zygocotyle lunata (adult)		12.39
Z. lunata (metacercariae)		7.09
Moniezia expansa (adult)	(*a*)	25.00
	(*b*)	34.13
Dipylidium caninum (adult)		16.09
Tetrabothrius sp. (adult)		41.33
Taenia taeniaformis (adult)		17.53
T. taeniaformis (cysticerci)		2.73

(*a*) and (*b*) refer to two different extractions.

DISCUSSION

Among invertebrate Metazoa, research involving serological reactions has been confined largely to helminths. One difficulty in per-

forming experiments on helminths and other lower invertebrates is that extracts of tissues must be used and they apparently are not so simple, chemically, as blood sera. Another complicating factor is that investigators have used different techniques, both in making the extractions and in determining titers, and therefore the results are not strictly comparable. Certain investigators have used the dry weight of the powdered whole worm as a basis for determination of the titer, but since there is considerable variation in the proportion of original powder which can be brought into solution, it is better to determine titers from the weight of substances actually in solution. Others have based titers upon protein content of the extracts, but this method presumes that all the nitrogen is active immunologically and ignores polysaccharides, which are also antigenic. Thus, in the present experiments, nitrogen determinations of the filtrate were not made because it was felt that the polysaccharides in solution were also important for antigenicity and specificity, and information concerning the amount of *all* materials injected would give a better index of the specific titers expected than an approximate knowledge of the amount of protein, determined from its nitrogen content.

Protein constitution has been and still must be emphasized as the *basis* for morphological as well as physiological characteristics. Upon this fundamental and basic protein pattern, on which *species* specificity depends, polysaccharides or other substances may be superimposed to confer either an altered species specificity or an additional *type* specificity, such as the type specificity of certain bacterial polysaccharides. Campbell (1937) noted that, although whole worm antigens indicated specific identity for the ascarids, *Ascaris lumbricoides* and *A. suum*, their polysaccharides were immunologically distinct. These two ascarids, therefore, may be considered two varieties or strains of the same species.

While proteins, presumably because of graded chemical differences, provide gradual differentiation in serological reactions and phylogenetic relations, the lipids of closely related species, or even in different organs of the same species, may differ considerably, and the lipids of distantly related species may be very similar, if not identical, immunochemically. The reason for the non-specific influence of lipids is therefore obvious, and, since they are antigenic when in combination with proteins, their complete removal is necessary in tests designed to determine zoölogical relationships.

The positive reactions noted by Fairley (1919–1933), by Cawston (1921) and by Höppli (1921) in tests between the several species of schistosomes and between *Fasciola hepatica* and *Schistosoma* materials

are to be interpreted as cross or aspecific reactions resulting from the lipids present in the extracts which were used. Such group reactions may be of diagnostic value but are unsatisfactory for determining relationships.

Lipids were present in the aqueous extracts of helminths used by Canning (1929), and he observed a *tissue* specificity, i.e., different tissues of the same species were immunologically distinct. The more general reactions obtained in tissues, such as the sperm where the largest amount of nucleoplasm was involved, and the more specific in the egg, where the largest amount of cytoplasm was present. Since lipids in different parts or organs of the same species may be of diverse immunochemical composition and since lipids in combination with proteins are antigenic, it is possible that the *tissue* specificity reported by Canning is directly attributable to qualitative and quantitative variation in the lipid content of extracts of different tissues, even though, in certain reactions, the proteins may have been identical. In accordance with the two-fold system of species specificity proposed by Landsteiner (1928), the reaction titer of antisera produced by the injection of hapten-containing antigens would be the resultant of two types of specificity, specificity of protein and that of hapten. In Canning's experiments, since antisera produced by tissues with a low lipid content, such as sperm, yielded more generalized reactions within a single species, it is possible that the primary protein specificity was not entirely nullified or concealed by a lipid specificity. The anti-protein fraction of the anti-sperm serum would be predominant and react with the protein component of egg antigen. However, since egg and sperm materials are both derivatives of reproductive organs, there is reason to expect chemical similarity of their lipid components. Thus, the anti-lipid fraction of sperm anti-serum would react to some extent with the lipid present in egg antigen. The resultant titer, then, would express reaction of both anti-protein and anti-lipid fractions of sperm antiserum with protein and lipid components, respectively, of the egg antigen. Injection of antigens containing moderate to large amounts of lipid would alter the specificity of resultant antisera, from one dependent on proteins to one dependent on lipid. Consequently, since the quantity of lipid in muscle is much less than that in egg and since the lipids of the two tissues may differ qualitatively, precipitation of the anti-egg serum could not be produced by muscle antigen (low lipid content) if diluted to the titer of egg antigen (high lipid content). The observations involving materials of different species suggested to Canning that "the cytoplasmic materials, if modified in consequence of inter-species variations, show a radical change, whereas nuclear ma-

terials, if modified, are changed only in a few factors with many others remaining identical." Since lipids do not exhibit gradual phylogenetic gradations, it is possible that the lipid of *Ascaris lumbricoides*, which alters or overshadows the protein specificity, may differ so radically from that of the acanthocephalan, *Gigantorhynchus gigas* (=*Macracanthorhynchus hirudinaceus*), that no cross reaction occurred when egg materials were employed, though the proteins are closely enough related, chemically, to give cross reactions, such as those noted with sperm materials (low lipid content). On the other hand, lipoidal substances present in the antigens of distantly related species may have yielded group reactions, since the presence of a common haptenic lipid in two different species evokes production of precipitins which will react with either antigen. If cytoplasmic materials manifest greater inter-species variation than nuclear materials, the lipids in the cytoplasm may be the variable factors.

Previous to Canning's observations, the comparative value and importance of cytoplasmic and nuclear materials had been considered by Mez (1926), who stated that nuclear materials are the same in all parts of a plant and at all times during the life cycle. Moreover, he proposed that nuclear proteins *only* are serologically important in relationship studies. According to Boyden (1936), this proposal is "clearly wrong as it applies to animals, at least, for we have excellent cases of serological reactions indicating relationships based on egg albumins and serum proteins which cannot be due entirely to nuclear proteins." The proteins and polysaccharides of both nuclear and cytoplasmic materials and, indeed, of intercellular materials as well, are immunologically important. Consequently, it is probable that use of lipid-free antigens, whether nuclear or cytoplasmic, will yield *species*-specific and not *tissue*-specific antisera.

Specificity of antisera is dependent on the method of preparation of the antigenic solutions used to evoke antibody production. Antigenic material should be extracted at *low* temperatures or, if *room* temperatures are employed, the number of labile substances which could be altered during the aqueous extraction should be reduced by previous removal of the alcohol-ether-soluble components. In Eisenbrandt's experiments (1936, 1938), although buffered solutions were employed, many labile substances may have been lost or altered, since extractions were carried out on *fresh* tissues at *room* temperatures, and the lipids were not removed to effect a reduction in labile substances. The chemical nature of materials prepared in this manner might be so greatly altered that the resultant antisera would yield neither accurate or consistent relationships. An altered chemical nature of the antigens

prepared by Eisenbrandt was evidenced by the fact that different antigens produced antisera whose homologous reaction titers varied from 1 : 2000 to 1 : 2,048,000. In fact, homologous antigens were restricted by Eisenbrandt to "those actually used for production of antibodies and are not used in a general sense to mean the same species." This restriction would seem unnecessary if the extraction of a single species yielded similar antigenic solutions in every case. The qualitative and quantitative variation in lipids which may have been present, the different chemical alterations in the different extractions, and/or the antigenic capacities of the resultant different lipid-protein complexes of even a single species, may account for the great range in homologous titers.

In the experiments reported by Eisenbrandt, reciprocal tests agreed with the original ones in only a single case, and the failure of reciprocal tests, according to him, "complicated the matter of interpretation and prevented the data from being of primary importance in this work." Furthermore, he stated, "The indications are that little or no emphasis can be placed upon reciprocal data when saline extracts of helminths are used as antigens in studying phylogeny unless further fractionation of the antigens be employed." Illustrative of the widely divergent results obtained by him, *Toxocara canis* and *Dirofilaria immitis* exhibited a 0.001 per cent relationship one way and a 50 per cent relationship when tested reciprocally. Boyden (1926) had stated that "it would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. . . . Only those values which check within the limits of error of the reaction may then be taken."

Even if they were not responsible for non-specific precipitin reactions, lipids should be removed from extracts, because, as has been mentioned, spontaneous precipitates, attributed to lipids, were observed by Eisenbrandt (1938) in certain antigenic solutions and by Mackie and Anderson (1937) in normal sera.

When the amount of material injected is determined by protein content alone, and this protein content is low, the large amounts of haptenic lipids and antigenic polysaccharides administered along with the proteins may cause prozone (antibody excess) in the antisera, and low titers would result because reactions between antisera and highly diluted antigen are inhibited by the antibody excess, as noted by Hooker and Boyd (1935) and Marrack (1938). Aside from the proteins, the qualitative and quantitative composition of the antigens employed by Eisenbrandt is not known, but the results indicate that the amount of haptenic and other antigenic substances injected, in the

cases of extracts having a low nitrogen content, produced antibody excess, which was responsible for some relatively low titers of the homologous reactions. A constant amount (0.0096 gram) of nitrogen was injected, but, since the nitrogen content of extracts varied considerably, equivalent amounts of antigenic material were not administered. For example, almost three times as much *Ascaridia lineata* as *Toxocara canis* antigen would be injected to administer equivalent amounts of nitrogen. The resultant increase in non-protein *A. lineata* materials injected, most of which would probably be antigenic, might cause such an increase in antibody content of the serum that, as a result of antibody excess, an inhibition of precipitation would occur when the higher dilutions of antigen were employed in tests. Another possible explanation for the low titers observed by Eisenbrandt in cases of low protein content of the antigens is that the simple haptenic substances actually inhibit precipitation because they occupy the combining sites on the antibody molecules, thus preventing combination of true antigen and antibody, as has been reported by Marrack and Smith (1932) and Haurowitz and Breinl (1933).

In relationship studies, uniform, consistent results are dependent primarily on the technique employed in preparing the antigenic solutions and in performing the tests with antisera. In the present work, it is possible that some degradation of labile substances occurred, since the aqueous extraction was performed at room temperatures, but the preliminary removal of lipids eliminates at least a part of the materials which may be altered during a twenty-four-hour saline extraction. The lipid concentration of the worms varied from as low as 2.7 per cent to as high as 41.3 per cent, so the antigenic capacities of the extracts, incident to the lipids present, would have varied considerably if antigenic solutions containing lipids had been employed. Indeed, the quantitative and qualitative differences in the lipids of adult and larval stages of the same species might have yielded serologically distinct antigens, especially if these lipids were altered during the extraction. Not only the lipids present in the extracts, but also the temperature at which the precipitin reaction is performed, the time at which the end-point of the tests is determined, and the clarity of the antigen and antiserum are factors which could affect the titer. The titers of comparable reactions at two different temperatures in this series of experiments were usually the same, but the reactions were more definite at 37° C. As reported by Boyden (1926) and by Eisenbrandt (1938), a reaction time longer than one hour is undesirable because the layer of precipitate becomes diffuse and begins to settle out and the end-point would be less precise. Boyden also noted that, after filtration of the

antiserum, the titer usually increased because of the increased clarity of the serum and the consequent greater ease of detecting a fine ring in the last tube, but, if the antiserum or the antigen is cloudy enough to note such a difference in titer, the original determination should not be made on unfiltered material. By working with clear reactants at 37° C., titers may be accurately defined and species differentiated.

In accordance with the chemical maturation which occurs in vertebrates, it seemed that differences between adult and larval helminthic materials might be detected. Contrary to expected results, very little, if any, difference was noted in the titers of larval and adult materials used in the present experiments, so, apparently, larval proteins are as well-differentiated as those of the adult; at least, larval and adult antigens are not serologically distinct. Species specificity of reactions was maintained in tests with all stages in the various life cycles studied. This fact is important for life history studies, since larval stages may be identified with their adult forms by serological methods. Improvement in technique may yield satisfactory reactions upon the injection of less antigenic material, and, since the amount of material available is very limited in most cases, especially the larval stages of species which parasitize marine animals, such technical improvement would be advantageous.

In the present research, a species-specific titer of 1 : 4000 resulted from injection of aqueous solutions containing only 40 mg. of dried antigen from which the lipids had been removed. As noted, high-titered antisera were produced when only 10 mg. of antigen were administered, but the results were not entirely consistent. The relation between the amount of antigenic material injected and the homologous titers of resultant antisera has varied extensively in experiments reported by different investigators. In most cases, however, much more antigen was injected than in this series of experiments. The observations of Wolfe (1939), combined with the results of present work, make it seem very probable that the technique can be improved further to yield consistent reaction titers with antisera produced upon the injection of small quantities of antigenic materials. One should not decrease the amount of antigen injected, however, at the expense of a homologous titer below 1 : 4000, because the margin by which to differentiate species-specific from group-specific reactions would be too small.

"Species" of helminths may be defined tentatively as a group of organisms the lipid-free antigen of which, when diluted to 1 : 4000 or more, yields a positive precipitin test within one hour with a rabbit antiserum produced by injecting 40 mg. of dry-weight, lipid-free anti-

genic material and withdrawn ten to twelve days after the last of four intravenous injections administered every third day.

The species which were studied in this series of experiments were not allocated to their positions in the classification until the precipitin tests had been completed. Upon determination of family and subfamily affiliations, a surprisingly good correlation existed between the titer of the precipitin test and the degree of relationship of the worms as determined from their development and morphology. Of particular interest was the serological relationship shown by material of *Cryptocotyle lingua* and *Siphodera vinaledwardsii;* the positive reactions in such relatively high dilutions support Manter's (1934) allocation of *Siphodera* to the family Heterophyidae,[1] to which *Cryptocotyle* also belongs. *Parorchis acanthus*, belonging to the family Echinostomatidae, is more closely related to *C. lingua* and to *S. vinaledwardsii* of the family Heterophyidae than to *Plagitura parva*, a member of the family Plagiorchidae, or to *Zygocotyle lunata* of the family Paramphistomidae, since positive reactions in much higher dilutions were obtained with the heterophid materials. A closer relationship was expressed by the reactions between *C. lingua* and *P. parva* than by tests involving *C. lingua* and *Z. lunata*, which indicates that Heterophyidae are more closely related to the Plagiorchidae than to the Paramphistomidae. Furthermore, the kinship between *C. lingua* and *Z. lunata* was closer than that between *P. acanthus* and *Z. lunata*, indicating that the Paramphistomidae are more closely related to the Heterophyidae than to the Echinostomatidae. A heterologous titer of 1 : 600 between *Moniezia expansa* and *Dipylidium caninum* denotes a closer relationship between these two cestodes than between either species and *Taenia taeniaformis*.

It will be recalled that no cross reactions occurred between materials of trematodes and cestodes which were tested. The explanation for the negative results may be found in one of two possibilities or perhaps a combination of both. The amount of antigen injected (40 mg.) may have been insufficient to evoke antisera which would exhibit a *visible* reaction with antigens of distantly related species, though some combination of antigen and heterologous antibody may have occurred. On the other hand, the high salt concentrations resulting from the evaporation of the antigenic extracts to the desired dilutions may have inhibited precipitation or caused a delay in the reaction so no precipitate was present at the end of an hour. For certain antigenic

[1] Price (1940), *Proc. Helminth. Soc. Washington*, 7: 1–13, transferred the subfamily Siphoderinae Manter, 1934, from Heterophyidae to the related family Cryptogonimidae.

concentrations employed in the tests, solutions were evaporated to at least one-tenth of their original volume. Evaporation to this extent would change the salt concentration from 0.15 N to 1.5 N, which would be sufficient to cause a delay if not complete inhibition of precipitation (cf. Landsteiner and Welecki, 1911; Eagle, 1932). To bring some of the solutions to the desired concentration of antigen, the salt concentration would have increased well above 2 N. Had the salt concentrations not been so high, positive reactions between trematode and cestode materials might have resulted in certain of these experiments.

An objective test which reveals and expresses quantitatively not only differences in empirical chemical constitution but also slight stereochemical differences, which are the basis for distinction of closely related species, is available in the precipitin reaction, since the results which are obtained are independent of individual interpretations. Boyd (1937) correlated the cross-reactivity of various hemocyanins with their chemical similarity, and Landsteiner and van der Scheer (1940) concluded that cross reactions between proteins of kindred species are ascribable to similarity and gradual variation in determinant structures. In the present experiments, cross reactions with the lipid-free antigens indicate that true specific relationships depend on differentiation of species-specific proteins, but it is possible that modifications may result from the presence of polysaccharides.

Summary and Conclusions

The value of the precipitin reaction as a method for determining phylogenetic relationships of helminths has been demonstrated, since the reaction differentiated species and expressed relationships which are in agreement with those derived from morphological comparisons of the species studied.

Uniformity of results may be attributed to the following factors: vacuum desiccation of worms kept in the frozen state, preliminary removal of lipids from desiccated material, control of hydrogen ion concentration during extractions and tests, control of temperatures at which reactions were performed, and knowledge of the *actual* amount of material in the antigenic solutions. Positive reactions were more definite and the rates of precipitation were more rapid at 37° C. than at room temperatures.

Antisera produced by injection of lipid-free antigens yield species-specific serological reactions whereas those evoked by lipid-containing antigens afford no better than group-specific tests. Therefore, preliminary removal of lipids from the desiccated antigen is necessary to exclude the possibility of group reactions. Cross reactions with lipid-

free antigens indicate that true specific relationships depend on differentiation of *species*-specific proteins, but it is possible that modifications may result from the presence of *type*-specific polysaccharides, since purified, protein-free polysaccharides are precipitinogenic.

Consistent results were obtained by injection of aqueous solutions containing forty milligrams of dried antigen from which the lipids had been removed.

The titer of a reaction is the highest antigen dilution which gives a visible precipitate when tested with an antiserum, the amount of antigen being determined on the basis of dry weight of antigenic material *actually in solution*. Heterologous titers never exceeded homologous ones, and they required a longer time for expression. Titers of all reactions in these experiments were confirmed by reciprocal tests.

Except for two species, *Cryptocotyle lingua* and *Siphodera vinaledwardsii*, the reactions were species-specific in antigen dilutions of 1 : 6000; in view of these exceptions, a titer of 1 : 4000 must be assigned to species specificity.

Analysis of precipitin tests involving various stages in the life cycles of the trematodes, *C. lingua*, *Parorchis acanthus* and *Zygocotyle lunata*, and the cestode, *Taenia taeniaformis*, revealed that larval and adult antigens are not serologically distinct. Serological recapitulation does not occur since larval proteins are as well-differentiated as those of the adult. Accordingly, serological methods may be used to identify the larval stages of a parasite with the adult and thus aid in working life cycles. Furthermore, since chemical maturity is reached early, both larval and adult stages of helminths could be used indiscriminately in making antigens either for diagnosis of helminthiasis or for establishing zoölogical relationships by serological methods.

The precipitin reactions confirmed, in general, the classification and relationships of the helminths as determined from developmental and morphological considerations. The tests demonstrated that the trematode families Echinostomatidae and Heterophyidae are more closely related to each other than to the Plagiorchidae or the Paramphistomidae. A closer relationship was expressed by the reactions between Heterophyidae and Plagiorchidae than by tests involving the Heterophyidae and Paramphistomidae, and the kinship between Heterophyidae and Paramphistomidae was closer than that between Echinostomatidae and Paramphistomidae. The titer of reactions between the cestodes, *Moniezia expansa* and *Dipylidium caninum*, denotes a close relationship between these two species which is not equaled by the titers of either species with *Taenia taeniaformis*. No reactions occurred between the trematodes and cestodes which were tested.

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THE FUNCTIONAL ORGANIZATION OF THE NERVOUS SYSTEM OF ENTEROPNEUSTA

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Introduction

The chordate affinities of balanoglossids have been widely accepted ever since Bateson advanced the suggestion in 1885 and have not seriously been challenged since Spengel (see especially 1893). Some of the most characteristic features of chordates relate to the nervous system. Moreover, the evolution of that group has outstandingly involved this system. It is of considerable interest, therefore, to inquire into the nature of the nervous mechanism of the Enteropneusta. Investigations of a purely morphological type were made by the earliest workers, but we lack, as yet, any conception of the functional stage of development represented or the plan of organization. The purpose of the present paper is to report some simple experimental evidence concerning the physiologic pattern of the nervous system in the Enteropneusta, and to offer a tentative generalized picture of its condition.

The intimate relation between receptors, nervous mechanism, and effectors renders it appropriate, in the case of animals whose general behavior is unfamiliar, to consider all three systems as an integrated unit. Since our only knowledge of the activities of the first two named systems is gained through observations of the overt activities of the effectors, the latter system is treated first.

The original observations contained herein relate chiefly to two eastern Pacific species, *Saccoglossus pusillus* (Ritter) (formerly *Dolichoglossus*) and *Balanoglossus occidentalis* (as yet a manuscript species). These were collected near San Diego, California, and the majority of the experiments were carried out in the laboratories of the Scripps Institution of Oceanography. It is a pleasure to acknowledge the kindnesses extended to me by Dr. H. U. Sverdrup and Dr. M. C. Sargent of that Institution.

Effectors and the Character of Response

The behavior of enteropneusts originates in at least three effector systems: ciliary, glandular, and muscular.

Cilia

Balanoglossids exhibit an extraordinarily high level of development of the general ciliature, and of dependence thereon. The entire surface of the body, external and internal (with very limited local exceptions), is provided with cilia which beat, in all probability, essentially continuously throughout life. Such an elaborate and extensive mechanism might be expected to perform more than such localized functions as the creation of respiratory or nutritive currents.

Removed from their normal habitat and placed in an aquarium for observation, specimens of *Saccoglossus pusillus* employ their cilia as the principal locomotor organs! True, parts of the body move, muscularly, relative to other parts; and progression may be accomplished by a series of "inch-worm" like movements involving extension and fixation of the proboscis followed by contraction of the trunk. But this does not occur regularly (Benham, 1899, describes it in *S. otagoensis* but does not indicate how usual it is), nor does any snake-like pushing against minute obstructions in the substratum. Peristaltic progression may be observed, but, under these artificial conditions at least, slow progress of the whole animal by means of cilia represents the chief method of locomotion.

Cilia are also used in building sand tubes and in burrowing. They can be directly observed in the former process. In *S. pusillus*, as Ritter and Davis (1904) and I have observed it, the proboscis cilia play the dominant rôle. The proboscis exhibits continual exploratory movements, its tip touching the substratum now here, now there. Every sand grain adhering to the sticky mucus thereon immediately begins to move backwards over the surface of the organ. Thus develops a stream of rapidly moving particles which, however, stop their advance at the level of the collar; here they accumulate to form a short sheath. By the force of the continual stream of particles from the powerful proboscis cilia, the sheath is pushed back, eventually to cover the entire trunk. The spectacular nature and the speed of this process not only emphasize the importance (mentioned below) of a sand tube but also suggest the probable rôle of the cilia in burrowing. For, doubtless, these organelles importantly contribute in this process, especially in the Ptychoderidae and Harrimaniidae where the extensor muscles of the proboscis are even more poorly developed than in the Spengeliidae (van der Horst, 1927–39).

Other functions of the cilia are entirely conjectural. Since not even a plausible guess has been offered concerning the function of the gill slits in balanoglossids, the significance of the highly developed ciliature lining those passages remains unknown. Whether the cilia

are of importance in an exchange of fluids between the coelomic cavities and the ocean through the proboscis and collar pores remains, with the antecedent question of the existence of such exchange, unproven. Notable as the only experimental evidence on the question is Bateson's (1885) demonstration that particles injected into the proboscis or collar coelom are ejected through the respective pores; but he found no intake of water, such as has frequently been thought to dilate the proboscis in the cycle of digging movements (e.g. Spengel, 1893).

Are the cilia of balanoglossids under nervous control? No work has been specifically directed at this point, but some observations are suggestive. The cilia beat, as would be expected, in the absence of nervous function, thus in anaesthetized animals and in the embryo before a nervous system has appeared. That is, ciliary activity is not dependent on the nervous system, a property generally ascribed to all cilia. The question which remains is: Can the nervous system modify the activity of the cilia? Since Parker's dictum in 1919, ". . . in fact, it may be stated that at present there is not the least ground for the assumption that true nervous activity is in any direct way involved in ordinary ciliary reactions," [1] there has been brought forward convincing evidence of nervous modification of ciliary activity in several cases (Göthlin, 1920; Alverdes, 1922; Copeland, 1922; Merton, 1923; Carter, 1926; McDonald, Leisure and Lenneman, 1928; Seo, 1931). It would seem from the evidence available that, over and above the intrinsic, epithelio-genic beat and coördination of all ciliary fields, some ciliary fields have come under nervous control. In such epithelia the normally continuous beat may be stopped, started, reversed or otherwise modified over the whole or only part of the field, at the "will" of the nervous system. Carter (1926) has set forth a rigid series of specifications for the thoroughly satisfactory demonstration of a case of nervously regulated cilia. No one has sought to satisfy these criteria for the balanoglossids, but, doubtless, many have observed, as I have, the striking behavior of the epithelium of adult enteropneusts in life. Here is seen an impressive degree of ciliary reversal and inhibition. Not whole fields but very small areas stop beating and start again many times in a few seconds, or only occasionally. Localized changes in direction of beat likewise occur. Now considerable, now limited areas of epithelium beat, now dorsally, now obliquely posteroventrally, dorsally again, anteriorly, anteroventrally, ventrally, shifting in rapid succession, the environment meanwhile remaining to the human senses uniform. Such remarkable behavior is difficult to explain,

[1] The qualification "ordinary" is puzzling. Nowhere in Parker's subsequent discussion does he refer to any extraordinary ciliary activity that might form an exception to his generalization.

in the absence of external stimuli, on the basis of an intrinsic epithelial mechanism acting entirely independently, but, of course, additional evidence is desirable before a nervous control is assumed. These features of ciliary activity were observed in *Saccoglossus pusillus* and only on the surface of the trunk, the proboscis cilia beating steadily posteriad.

It would seem probable, then, that the unusually extensive and elaborate ciliary mechanism of balanoglossids is of profound importance to the economy of the animal, at least in certain kinds of locomotion, in sand tube formation and burrowing. The occurrence of specialized local ciliary apparatus and the complex behavior of certain ciliary fields indicate that it is probably important in other more specific and complex functions. The occurrence, speed, and localization of inhibition and resumption as well as reversal of ciliary beat suggest that the present animals may offer a new case of nervous control of cilia.

Glandular Activity

Enteropneusts are among the most copious producers of mucus known. Furthermore, to reason from the variety of histologically differentiated types of unicellular epithelial glands, they must elaborate a considerable array of different substances. The nature, importance, and modifications in secretion of these substances remain virtually unknown. From the highly developed state of the mechanism, the volume of its production and the habit of the animals, it seems safe to regard this system as of great importance. But no evidence can be cited bearing on the question of control of glandular activity beyond the facts that anaesthetized worms and isolated strips of epithelium continue to secrete. Probably largely independent of the nervous system, the existence or nature of direct or indirect nervous control cannot as yet be surmised.

Light Production

Several species of balanoglossids have been reported to produce light upon stimulation after dark-adaptation (Kuwano, 1902; Crozier, 1917). The nature of the phenomenon has not been determined in these animals. Crozier assumes that it is intrinsic, i.e., that the light is produced by the worm itself, and it may be that some of the plethora of unicellular integumentary glands are responsible. In this case, of course, the phosphorescent organs should not be listed as a distinct effector system. The only facts known about the phenomenon are that it is elicited by external stimuli such as poking the worm or tapping the aquarium, that it is inhibited by exposure of the subject to light,

and that it is accomplished as well by small fragments as by intact animals. Regarding the question of nervous control, Crozier argues from the last-named fact that light production must be independent of the nervous system. But, in view of the high degree of neuromuscular autonomy of even small fragments, demonstrated later in this paper, his position is no longer tenable, and the first two facts named above suggest reflexes involving the nervous system.

Muscular Activity

The normal habit of life of all enteropneusts is doubtless benthonic. Many species are burrowers. Their food habits though not yet elucidated are clearly passive, for in general these animals are sand-eaters and obtain their nourishment from organic matter contained in the substratum. Their means of protection, as well, seem likely to prove passive, though even less understood than the means of nutrition. Very possibly the well-known iodoform odor of many species functions in this connection and perhaps we may thus account for some of the diverse integumentary glands.

Corresponding to such a passive mode of life, balanoglossids are among the most sluggish of all free-living animals. Not only are their movements characteristically sluggish but the observer is struck by the small number of different responses possible. The movements incident to burrowing and to advance and retreat within the burrows, comprise the sum total of muscular exertions of most of these animals.

Cases of an extraordinary degree of activity for enteropneusts are the reports of Ikeda (1908) and Spengel (1909) of the pelagic occurrence of two species of the genus *Glandiceps*. Swarms of individuals were encountered swimming at the surface under conditions which could not be correlated with breeding phenomena or any other obvious factor. The mechanism of swimming is not made clear.

In view of the frequent misconceptions thereof, a word may be said concerning the mechanism of burrowing. Most importantly, it is not yet clearly understood, even in essential respects. The use of the coelomic pores for dilation and collapse of the proboscis and collar with sea water has not been demonstrated and is, on several anatomical grounds, unlikely. The service of the "notochord" and its secreted skeletal sheath is principally that of the muscle attachment, secondly that of strengthening the proboscis-collar junction; but it is not to be thought of as providing an otherwise flabby proboscis with the rigidity required for digging in heavy mud and sand. For that organ, in short- as well as in long-proboscis species, *is* flabby, and balanoglossids burrow slowly and inefficiently because of their lack of rigidity. The muscles principally responsible for forcing the proboscis into the sub-

stratum, i.e., the extensors of that organ, are the circular muscles which are conspicuously poorly developed in all species, and especially so in the Harrimaniidae and Ptychoderidae. Much more voluminous are the longitudinal muscles which shorten and, incidentally, swell the proboscis. (The disproportion is even greater in collar and trunk.) These facts emphasize the probabilities that: (1) those balanoglossids called, above, "burrowers" are chiefly livers *in* (more or less permanent) burrows, not active diggers, and (2) cilia are very likely of great importance in burrowing, as in sand-tube formation (very likely in the same way) and in locomotion.

To emphasize the paucity of clearly-defined and diversified movements, the results of the search for responses lending themselves to physiologic testing in the experiments described below may be cited. Using *Saccoglossus pusillus*, I was able to find but a single reflex even approaching an adequately dependable and clean-cut piece of behavior for which to test experimental subjects. This is elicited by tactile stimulation, for example, a gentle poke with a probe, applied to the proboscis. If each of a series of conditions having to do with the state of the specimen has been satisfied, such a stimulus results in the most rapid muscular response this animal is capable of, namely, a contraction of the longitudinal muscles of the trunk. The trunk is thereby shortened and coiled, but the collar and proboscis need not be involved in the movement at all.

The predictability of this "shortening reflex" depends most on the following conditions. The specimen must be in good condition, uninjured in collection except as regards the abdomen, which may even be missing without affecting the response. It must have been but recently removed from its burrow, whether in mud flat or aquarium. It should be active, moving about the vessel in exploratory fashion; the best responses are obtained from animals stretched out straight, in forward, doubtless ciliary, progression. And, finally, it must not have executed a generalized contraction within the preceding five minutes.

This is, in all probability, a true nervous response, since it takes place at a distance from the point of stimulation and is abolished, reversibly, by light anaesthesia such as does not impair ciliary or glandular activity or irritability of the muscles to strong direct stimulation.

We have here to do with a motor apparatus consisting of smooth fibers, not segregated into discrete muscles but occurring in loose masses or layers. It is functionally characterized by sluggish action, generalized responses, a lack of differentiated reflexes, and a relatively simple rôle in a rather passive, benthonic habit of life.

Receptors and the Known Adequate Stimuli

Although several authors have suggested a sensory nature for certain peculiar structures of the integument (Spengel, 1893; Assheton, 1908; van der Horst, 1930; Brambell and Cole, 1939), the lack of adequate reason for so regarding them makes it necessary to say that as yet no sense *organ* has been demonstrated in the Enteropneusta. This is a very astonishing fact, considering the early origin of differentiated organs of reception, and indicates not only a very simple type of receptor mechanism, but the probability of a very generalized type of irritability. The receptors are assumed to be scattered sense cells in the epithelium. Only one author has claimed to have seen sensory cells in balanoglossids, Hess (1938) having identified certain large basiepithelial, bipolar cells as photoreceptors.

The facts known about sensory physiology in enteropneusts fall into three groups according to the nature of the stimuli: mechanical, chemical, and photic.

Mechanical Stimuli

The irritability to probing and to tapping of the aquarium has already been mentioned. Characteristic responses, varying with the species, result from the stimulus of contact with a sandy or muddy substratum.[2] Species-characteristic behavior in response to exposure by the tide is also reported. This involves retraction or protrusion of the proboscis, or migration up or down in the burrow (Ritter and Davis, 1904; Morgan, 1894; Assheton, 1908; Stiasny, 1910). Disturbance of the substratum, as by footfalls, brings about quick retraction in the burrow.

Chemical Stimuli

We know virtually nothing concerning the sensitivity of balanoglossids to dissolved chemicals. The single report on record is the statement of Crozier (1915) that "for the chlorides of the alkaline metals, the normal lyotropic series, $K > NH_4 > Li > Na$, was found to express their stimulating efficiency; this was mainly a kation stimulation, but in the case of salts ($CaCl_2$, e.g.) which did not stimulate strongly, other anions (in this case $Ca(NO_3)_2$) were effective as stimulating agents." Crozier, further, was able to separate, by exhaustion or anaesthesia, the mechanical-chemical irritability from photic sensi-

[2] The curious response of *Saccoglossus pusillus* to the absence of mud is death, within as short a time as two or three days. Whether this is due to starvation, exhaustion or some other factor has not been determined, but the suddenness of death and the rapidity of cytolytic disintegration even in well-aerated, darkened aquaria are spectacular.

tivity, but the two former types of irritability could not be physiologically separated. He concluded that there are generalized receptors open to both mechanical and chemical stimulation, but recognized that these facts are "insufficient proof of the separateness of the sensory organs concerned in the reception of photic stimulation." He felt, however, that they do show "that photic irritability depends upon a process distinct from that implicated in the other modes of stimulation."

Photic Stimulation

Crozier noted in 1915 that *Ptychodera* and *Glossobalanus* showed "differential sensitivity" to light, and were indeed negatively photokinetic. Pursuing this form of experimentation, he found (1915, 1917) that: (1) The sensitivity to light was enhanced by dark-adaptation. (2) Photic and other forms of irritability could be readily separated physiologically (see above). (3) The collar nerve cord is not necessary for the kinetic response to light. (4) Posterior fragments or pieces of abdomen, including the caudal tip, are able to respond, but move away from the source of light tail first! (5) The tip of the proboscis is most sensitive, although the whole body exhibits some irritability. (6) Exposure to light greatly inhibits the ability of these animals to produce light, though such production could always be brought about by moderate induction shocks. (7) Even small fragments are able to produce light and are inhibited therefrom by exposure to light.

The only other experimental work upon enteropneusts has been that of Hess, whose interest was primarily in the photic response of these animals. Hess (1931, 1936, 1937, 1938) confirmed the observations of Crozier and then attempted to analyze the response by removing various regions of the body. He reported that the response (orientation and movement away from the source of light) was unimpaired by cutting off the proboscis, or the dorsal third of the collar (to remove the collar nerve cord), or its ventral third (to remove the "ventral nerve cord" of the collar [a cord which does not exist]), or the trunk and proboscis (leaving only collar and "peduncle" or proboscis stalk). The isolated proboscis, likewise, was able to respond. But, pieces from which the proboscis and its peduncle had been removed (or proboscis, peduncle, and collar, i.e., isolated trunk fragments) were unable to orient with respect to light. He concludes that the peduncle contains a "nervous center" concerned with light reactions, a center of which the proboscis, unlike the rest of the body, is independent. In *Ptychodera bahamensis* Hess considered the reactions sufficiently clean-cut to take reaction times. For all regions or combinations except the isolated proboscis these times were very nearly the same (1.90″–2.77″),

including the, presumably, optimal combinations—peduncle, collar, and trunk—as well as abdominal fragments which cannot orient but only, it is to be inferred, squirm aimlessly. The reaction time of the proboscis is given as 25.23″. This remarkably slow result Hess explains as being due to the fact that the proboscis has now been severed from the "central nervous system." That this "most sensitive region of the body," which can, when isolated, orient and move away from light, should lack this nervous ability—a rapid light response—which is possessed by a trunk fragment, is difficult to believe. His interpretation (or the figure for the reaction time of the proboscis) seems the more dubious (1) in view of the concept, arrived at below, of the nature of the "central nervous system," (2) in view of the independence of the light response of the great nervous concentrations in any case (Crozier, 1917, and below), and (3) in view of the general behavior and properties of the proboscis.

In 1938 Hess repeated on *Saccoglossus kowalevskii* those experiments involving intact animals. Again, intensities of light above a certain minimum resulted in negative orientation with little tendency to trial and error movements; but weak illumination produced a tendency to orient toward the source. Pin-point light exploration showed all regions to be sensitive, but some regions more so than others, the proboscis being the most sensitive.

My own experiments with photic stimulation were performed on *S. pusillus*. This species was found to be definitely negatively tropic although its response was very soon lost after removal of the animals from mud and exposure to light. The photic response is abolished by light anaesthesia, but returns after brief exposure to clean sea water. This shows, following Parker (1919), that the reaction depends upon the nervous system.

The methods of investigation used by Hess on *Ptychodera* presuppose an extended, straight animal, relatively motionless before exposure to light, capable of turning promptly upon exposure in a definite direction and without trial and error or "indecision." *S. pusillus* rarely assumes any but a coiled position with only the proboscis relatively straight, almost continuously moves about in exploratory fashion if fresh and healthy, and responds, often (especially in specimens removed from mud for some time, as in operatives and their controls which are rested in a constant temperature darkroom for at least one hour) with considerable hesitation. These differences render inapplicable to *S. pusillus* the technics of Hess, especially the determination of reaction times. But the existence of photic irritability was clear and the regional assignment of responsibility for the negative orientation to light was quite possible.

Hess's (1937) method of analysis was repeated upon variously constituted fragments of the body. After operation these fragments were dark-adapted at 20° C. for 1 to 2 hours and then exposed to a laterally incident beam of light with the following results. (1) Isolated proboscides, cut well forward of the peduncle, can regularly turn away from the source of light. (2) As would be expected, fragments consisting of proboscis and peduncle or of proboscis, peduncle, and collar readily respond and orient themselves in the same way. (3) Specimens with the collar mutilated, either by cutting across or removing the entire dorsal third (to include the collar nerve cord) are perfectly able to orient with respect to light. Controls with the ventral third of the collar missing or transected respond similarly. (4) Fragments of trunk region are incapable of orientation with respect to the direction of illumination. (5) Pieces consisting of collar and trunk but without peduncle or proboscis are incapable of orientation. Thus far all results correspond exactly with those of Hess. But (6) pieces similar to the last-named with the addition of the peduncle, i.e., with nothing removed but the proboscis, never succeeded in turning away from the light source in these experiments. The operations in this case were carefully performed so as to leave intact the entire peduncle and base of the proboscis, since this result is diametrically opposed to that reported by Hess for *Ptychodera*. It was found, by depriving further specimens of *Saccoglossus* of various fractions of the proboscis, that nearly one-third of its length must remain attached to the peduncle to obtain occasional definite responses, and more than one-third for consistent negative phototropic movements. It must be recalled that *Saccoglossus* is characterized by an extremely long proboscis; when two-thirds of it has been removed the remainder still exceeds the collar in length and is, proportionally, longer than the intact proboscis of *Ptychodera*. The long proboscis permits that much more exact delimitation and transection. The present conclusions, therefore, may be stated thus: although all regions of the body are sensitive to light, responding by aimless squirming, only the proboscis is capable of orienting with respect to the source of light, and the normal response of the intact animal is dependent upon that organ. The assumptions of a "center" in the usual neurologic sense, or of a central nervous system are not justified by the evidence at hand. Hess's results are open to the same interpretation, if it be supposed that his operation corresponding to (6), above, left a minimal amount of the (very short) proboscis still attached to its peduncle to orient the preparation to light.

The general nature of the sensory physiology of balanoglossids is,

therefore, clear, though the details are only sketchily indicated. Generalized receptor cells are scattered throughout the integument though differentially concentrated. The proboscis is most sensitive and, in a general way, a gradient of decreasing sensitivity from anterior to posterior exists. The senses of enteropneusts are at a very low level of development and it would seem probable that the animals are not greatly dependent on them. This condition may be correlated with the essentially passive, bottom-living habit of life. Though sensitive to a variety of forms of stimulation, the few and generalized

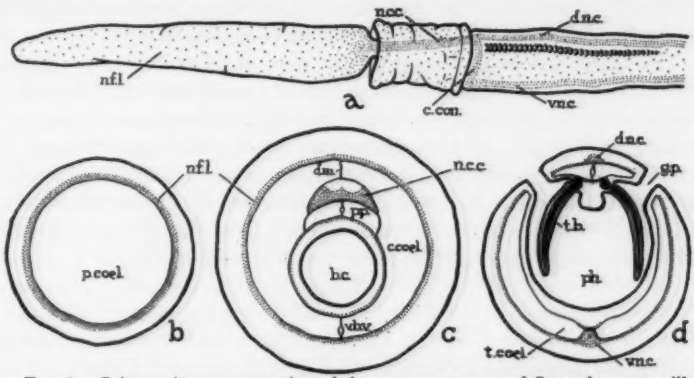

FIG. 1. Schematic representation of the nervous system of *Saccoglossus pusillus*. The nerve fiber layer of the epithelial nervous system is indicated by stippling. (*a*) Lateral view of proboscis, collar, and anterior end of trunk. (*b*) Transverse section of proboscis, (*c*) of collar, (*d*) of pharyngeal region of trunk. Organs occupying coelom have been omitted. *b.c.*, buccal cavity; *c.coel.*, collar coelom; *c.con.*, circular nerve connective; *d.m.*, dorsal mesentery; *d.n.c.*, dorsal nerve cord of trunk; *g.p.*, gill pore; *n.c.c.*, internal nerve cord of collar; *n.f.l.*, nerve fiber layer of superficial plexus; *p.coel.*, proboscis coelom; *ph.*, pharynx; *p.p.*, perihaemal pocket of trunk coelom; *t.b.*, tongue bar (coelom not shown); *t.coel.*, trunk coelom; *v.b.v.*, ventral blood vessel; *v.n.c.*, ventral nerve cord of trunk.

kinds of responses suggest a low order of differential recognition of stimuli, and little or no differentiation of "specific nerve energies." The beginnings of complexity are seen, for instance, in the dependence of the whole upon one part for orientation to light, but on the whole the level that has been achieved in this group is lower than that of many of the coelenterates—the simplest animals having any nervous system.

FUNCTIONAL ORGANIZATION OF THE NERVOUS SYSTEM

Anatomically, the nervous system of balanoglossids, as described by its discoverer (Spengel, 1877, 1893, and not since significantly modified), consists (Fig. 1) of a general superficial plexus located at the

base of the outer epithelium everywhere over the body and locally thickened to form so-called cords in the mid-dorsal and mid-ventral lines of the trunk—through the dorsal coelom of the collar as the only internal part, and again on the surface of the peduncle. All of these regions of nervous concentration, except the collar cord, are continuous with and connected by the general plexus; thus the peduncular thickening and the proboscis plexus, the dorsal and ventral trunk cords and the lateral trunk plexus are continuous basiepithelial layers. The ventral cord is confined to the trunk and at its anterior end a circular connective, traversing the collar-trunk boundary, passes from it to the dorsal cord where that is continued into the collar coelom.

The most important feature of the neuromuscular organization of these animals, and one profoundly conditioning the character and possibilities of experiment on it, has already been emphasized, namely, the simplicity and lack of variety of responses. Not only this, which could be said of an animal with a single, clean-cut, invariable response, but the responses of *Saccoglossus pusillus* are not uniform and dependable. The single sufficiently predictable reflex found in this species has been described above as the "shortening reflex."

The Nerve Cords

If the dorsal nerve cord of the trunk in a healthy specimen of *S. pusillus* be transected a little behind the collar (the technic of the operation and the extent of the cut make virtually no difference; a single stroke with sharp iridectomy scissors was found most convenient), a preparation is obtained in which the shortening reflex is hardly impaired. Often it is less certain, sometimes slower, but many responses are obtained which cannot be distinguished from those of normal subjects. Using *Balanoglossus occidentalis*, a large species only occasionally obtainable, this result is even clearer. It must be said that all experiments with this species were performed upon larger or smaller fragments, since I have never seen an intact specimen. However, it is easily demonstrable with other species, and has indeed been well known to earlier workers, that loss of the posterior part of the trunk has no noticeable effect on behavior. With this species it is possible to show, not only a nearly normal posterior response to anterior stimulation, but also a proboscis response to trunk stimulation virtually unaffected by transection of the dorsal nerve cord.

Similar operations affecting the ventral cord, however, have a marked effect on the shortening reflex. It is greatly impaired posterior to the level of the cut, is slower, prolonged as sluggish writhings, and has a higher threshold of stimulation. Again, the results with

Balanoglossus are even more clean cut. The animal ceases its spontaneous exploratory movements, but spontaneous peristaltic waves pass back from the level of the cut, as in isolated trunk fragments.

The effect of transection of both cords is even more pronounced. Now the response is virtually abolished posterior to the level of the operation. Stimulation adequate for the controls is entirely ineffective. Stronger prodding of the proboscis may elicit contraction of proboscis, collar, and trunk muscles anterior to the cuts, and a few of the contiguous muscles immediately posterior thereto. Repeated, relatively strong stimulation does, however, invariably produce movements of the abdomen, although not of the same character as the normal rapid shortening.

Lateral incisions, in the frontal plane, at the junction of trunk and collar, one on each side, yield results similar to the ventral cord transection above. Such incisions interrupt the continuity of circular connectives which thus appear to be of considerable importance in normal communication between the proboscis and the ventral cord of the trunk; that is, the alternative path through the general lateral plexus is not the route normally employed in rapid long distance reflexes. If the dorsal cord of such an individual be severed, the properties of the resulting preparation are precisely those described in the last paragraph.

Transverse cuts through the dorsal third of the collar will include the collar nerve cord. Such an operation results in animals markedly less active than normal, resembling in this feature and in the greatly impaired shortening reflex animals with both trunk cords interrupted.

It is curiously difficult to observe a ganglionic or modifying function in the great cord of the collar which, from the anatomical evidence, has been thought to be a central organ. I have failed as yet to devise an experiment to demonstrate such a function. Its strategic and unusual position, along the path of the main reflexes, crucially affects its accessibility to experimental analysis. The great nerve centers of other animals are usually terminal, but here we are unable to isolate the ganglionic function without interrupting the main nerve paths that pass through the collar cord. It is perhaps significant that nothing more can at present be said than this: No effect of cutting the collar cord has revealed itself except such as can be explained as a parting of pathways. Thus burrowing and photic reactions are quite normal after collar cord section (or removal); but the shortening response of the trunk to proboscis stimulation is impaired in the same way that it is after interrupting the cords of the trunk.

To test the effect of the operations *per se*, aside from the interrup-

tion of nerve cords, I have made various mutilations of the body wall in regions not traversed by concentrations of nerve tissue. These have no detectable effect on the ability to jerk up the abdomen upon gentle proboscis stimulation. However, more drastic operations, such as removal of the anterior third or half of the proboscis, have a noticeable effect, at least temporarily. Such subjects show a decrease in spontaneous movement, especially exploratory progression, and are rarely stretched out straight, but remain coiled and quiet. These characteristics are relative; no profound change is found in the subjects in question. They show no loss in ability to respond normally to proboscis stimulation.

Section of the cords in isolated fragments of trunk region reveals a further detail of their function. Whereas the branchio-genital and

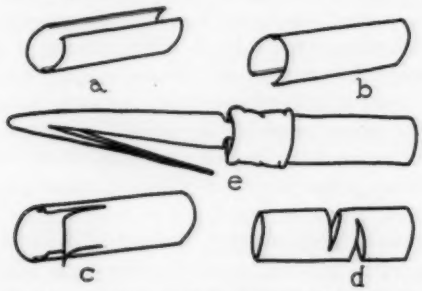

FIG. 2. Diagrams of operations on fragments of balanoglossids. See text for explanation.

especially the abdominal regions of intact animals exhibit almost continuous writhing movements, a length of trunk cut off from collar and proboscis lies motionless for many seconds or longer. Moreover, the occasional waves of contraction which do initiate in such pieces (especially branchial and terminal regions) or the slow peristaltic waves elicited by external stimuli are interrupted by interruption of the cords. If both cords are cut, only a few muscles immediately behind that level contract. If only one cord is cut, the waves are stopped, but not as sharply. Occasionally such a contraction wave starts up again after a delay of one half second or more and progresses to the level of the next cut. The ventral cord appears to be more influential, the dorsal somewhat less important, although the evidence for this difference is not here as clear as above.

A curious difference between dorsal and ventral nerve cords is revealed by the following experiment. This, further, anticipates the

next section by demonstrating that impulses are conducted through the lateral plexus, in this case between the cords in the transverse plane. A length of trunk region is opened by a mid-dorsal longitudinal slit (Fig. 2, *a*) and stimulated anywhere along the cut margin of one side. Moderate stimulation suffices to elicit not only a muscular response on the same side but also contralaterally. An even weaker stimulus may produce an ipsilateral contraction alone. The reciprocal preparation, however, opened by a mid-ventral slit (Fig. 2, *b*), will not, even upon strong stimulation, produce a contralateral response, although contractions of the same side are easily aroused. These facts indicate some difference in the pattern of the nerve cords such that impulses readily cross the ventral but not the dorsal cord. The ability to pass across the midline would seem, *a priori*, to be primitive; on this assumption the dorsal cord is more specialized than the ventral, although, as indicated by the previous experiments, the latter is the more important.

Such are the effects manifested in the first few hours after operating. What, now, happens to such operatives when kept, undisturbed, under favorable conditions, for some days? Of a group of twelve specimens so treated, nine recovered normal ability to respond within ten days, four of these being normal by the fifth day. Of the remaining three, two died and one recovered its active behavior but not its normal, rapid response. Among the animals which recovered completely were subjects which had suffered transection of the collar cord and others whose dorsal and ventral nerve cords had been interrupted in the trunk. Complete tissue regeneration had not occurred, but, histologically, fibrous continuity of the cords was reestablished to a degree which was evidently sufficient to reestablish functional paths even in these short periods.

Experiments on the General Plexus

It has already been indicated that the body wall aside from the nerve cords is able to conduct nervous impulses. This, it seems logical to assume, probably takes place through that thin basiepithelial nerve fiber layer which has been referred to as the general plexus. The present section is devoted to an elucidation of its properties.

Diffuse Transmission.—The experiment with trunk segments slit open longitudinally demonstrated dorso-ventral conduction (actually involving an arc in the transverse plane). By stimulating first one side and then the other of the first-described preparation, it can be seen that the lateral plexus is conducting now dorsally, now ventrally. The threshold of stimulation for conduction in this direction is, furthermore, low relative to that in the next several experiments.

It was noted above that, after section of the great conduction tracts (collar cord or both trunk cords), persistent, strong stimulation of the proboscis will elicit contraction of the abdomen, although this contraction is delayed and abnormal in character. This suggests the existence of alternative, though poorer and slower paths. The general plexus can, therefore, conduct anteroposteriorly. In *Balanoglossus occidentalis* it was possible to demonstrate conduction anteriorly as well as posteriorly.

Such behavior is highly suggestive of a nerve net, for the property of diffuse transmission is characteristic of such nervous systems and is not found in others. The anatomical relations, as far as known, correspond with those of typical nerve net systems but the distinguishing features of the nerve net are more important physiologically than anatomically; moreover, the functional characteristics are more easily and certainly ascertained than the crucial histologic relations.

As illustrative of a number of other experiments demonstrating conduction through the lateral plexus, the following may be described. A long piece of trunk region is incised longitudinally just lateral to the dorsal cord and again just to one side—the same side—of the ventral cord, the incisions extending through about half of the length of the piece. There is thus produced a preparation (Fig. 2, *c*) from which projects a tongue of lateral body wall with no nerve cord, still attached, however, to the remainder of the trunk in which the cords are intact. The cordless tongue being now strongly stimulated, a delayed bilateral response in the uncut posterior end follows. This is much clearer in *B. occidentalis* than in *S. pusillus*, doubtless because the former's larger size permits a neater operation. In this species the response even extends forward into the unstimulated anterior region. Stimulation of this region, provided as it is with intact cords, produces prompt contraction of the posterior end, and need be only a fraction of the strength or duration of the stimulus required in the case of the cordless tongue. If the cords then be severed at the base of the tongue, the anterior region still possessed of cords will behave precisely as did the cordless tongue in respect to higher threshold and delayed conduction.

Again, a trunk fragment may be deeply notched, twice, transversely, one incision extending from the dorsal side through at least two-thirds of the animal's diameter, the other from the ventral side and about 5 mm. in front of or behind the first (Fig. 2, *d*). Strong stimulation anterior to the operations will elicit posterior responses, indicating the conduction of impulses around the overlapping obstacles, but with considerable difficulty. Occasional spontaneous contractions of these pieces were noticed. Beginning near one end

these spread as waves, apparently unimpeded by the cuts, to involve the whole fragment in contraction at the same time.

In all of these and other similar experiments it was noticed that much greater difficulty is encountered in anteroposterior transmission than in dorso-ventral. Much stronger stimuli are required for the former than for the latter.

The proboscis is especially favorable for demonstrations of diffuse transmission by reason of the absence of differentiated conduction paths. The proboscis may be slit nearly in two in any direction and yet respond as a whole to adequate stimulation of either half. This statement must be qualified since the proboscis does not respond to stimuli in the simple, certain manner of other parts, although it is the most sensitive region. It is as likely to continue its aimless motions as to react clearly to a prodding which suffices for the jerking up of the abdomen. The most dependable form of this experiment takes advantage of the fact that response of the posterior regions to proboscis stimulation is more reliable than its own response. The proboscis is cut obliquely, from the posterior margin toward a point on its main axis near the anterior end and in any plane about that axis (Fig. 2, *e*). The stimulus is then applied to the posterior end of the free tongue. The impulse is propagated anteriorly, around the cut, and then posteriorly to elicit a trunk shortening. Or the same experiment can be executed on preparations with the proboscis transversely and deeply notched, from any side, showing the absence of special conduction paths and the ability of the plexus to conduct impulses over whatever fraction of itself remains intact.

But the simplest demonstration that transmission may be truly diffuse utilizes fragments of isolated proboscis, thus eliminating any possible central nervous influence. Such fragments may be stimulated at any given point by a single contact stimulus and the response observed to take place all around this point and to involve a rapidly widening circle, i.e., impulses are being conducted in all directions away from this spot. Another stimulus at a neighboring point elicits the same behavior. But now some of the same paths are being traversed in the opposite direction. An infinity of different points may thus be stimulated with the same result and impulses shown to pass in both directions between any two of them.

Conduction with Decrement.—It is especially easy with the proboscis of *B. occidentalis* to demonstrate that the response to a stimulus spreads in every direction from the point of stimulation but only to a certain distance. Furthermore, this distance varies according to the strength of the stimulus, so that a larger area is called into muscular

contraction by a stronger stimulus. Again, we find a property characteristic of typical nerve nets (Parker, 1919) manifested in balanoglossids.

Autonomy.—The parts of the body of balanoglossids are remarkable for the degree of autonomy they exhibit. This property is highly significant from the point of view of the organization of the nervous system and we may describe its manifestations in isolated fragments of these animals.

Isolated proboscides immediately strike the attention by reason of their activity and irritability. They live well for many days. (I have never had opportunity to keep fragments long enough for any considerable amount of regeneration.) They respond to touch and to light as well as in the intact worm, move about in an exploratory manner—by means of cilia, and contract vigorously. But they are not as successful as are whole animals at burrowing, possibly for lack of weight and length in obtaining a purchase on the substratum. The most clearly autonomous major region, the proboscis, behaves alike attached or unattached to collar and trunk.

The isolated collar fragments which I have observed lived but one or two days before disintegrating. They were capable, however, of responding locally to touch and light. This region performs few muscular movements itself either in the intact animal or when isolated and most of its organs are of significance chiefly for the whole organism (collar cord, great blood vessels, buccal apparatus, specialized integumentary glands, skeleton). It has, nevertheless, the neuromuscular equipment to respond autonomously to stimuli.

Isolated trunks or parts thereof seem quite normal. Characteristically responding only by localized contraction of the spot stimulated, they exhibit generalized contractions only occasionally. If small enough, they progress by means of cilia; larger pieces do not progress at all. Sand is ingested and egested but burrowing is not effected. Longevity, in my specimens, was not as great as that of proboscis fragments, although Davydov (1908) and others have kept such fragments long enough for complete regeneration.

Thus, all regions possess the mechanism for nervous reflexes, and moreover, respond very largely by muscular contraction of very small areas immediately about the point of stimulation. It is not surprising, therefore, though crucially significant as an indication of the nervous pattern, to find that smaller and smaller fragments of the body wall are quite able to react nervously, the smallest piece that I could neatly cut and observe—having approximately 1 sq. mm. of surface—displaying nervous irritability and true reflexes. The evidences

against regarding this as direct muscular stimulation are: (1) the strength of adequate stimuli, which need be no greater than those required for indubitable, long distance reflexes in the intact animal whereas direct stimulation of muscles (thus in narcotized specimens) requires very strong stimuli, and (2) the reversible disappearance of the reflexes in light narcosis. Finally, it should be stated that fragments from any part of the surface of any region (except areas where the body wall is devoid of musculature) may thus react autonomously.

If small pieces of an animal, from any region, are able to execute normal reflexes, it is evident that the impulse does not need to travel to a distant central organ and then back to an effector, but that the entire path of the reflex is located within that small piece. Autonomy of this type is highly characteristic of nerve net-equipped organisms and its presence in balanoglossids is convincing evidence that the general superficial plexus of these animals is essentially a nerve net.

Polarization.—One of the earliest modifications of the primitive nerve net was in the direction of acquiring polarity. This means that impulses will travel more easily in one direction than in others, and will tend to spread differentially along an axis instead of uniformly in an expanding circle. It is of interest to look for evidence of polarity in the diffuse plexus of enteropneusts.

A proboscis cut into two pieces, transversely, exhibits no difference in the behavior of the two cut faces. The pieces themselves show no evidence of polarity aside from the direction of progression which is a result of the polarity of the ciliated field. It has already been shown that impulses spread in all directions from the point of stimulation in the proboscis.

The trunk exhibits an obvious polarity traceable to the nerve cords, but in the absence of these structures the lateral plexus shows little evidence thereof. Evidence has already been cited, however, which indicates a differential orientation of pathways such that impulses pass easily in the dorso-ventral direction, but only with difficulty longitudinally. No experiments have been devised to show that these two axes are heteropolar, although it is to be expected from other, already cited facts. The poles of the dorso-ventral axis would be the dorsal and ventral cords. These differ in several ways, not only histologically, but functionally, thus in respect to passage of impulses across the midline and in relative importance for the normal responses of the intact animal. Along the anteroposterior axis the usual direction of propagation of waves of contraction is from anterior to posterior. How much this normally depends on the cords cannot yet be

said, but it is possible that a degree of anteroposterior polarity exists in the plexus.

Action of Drugs

Unfortunately, almost no opportunity has been found to experiment with drugs whose effect on the nervous systems of higher animals is known. But two observations may be mentioned as of some potential interest.

The anaesthetic action of $MgSO_4$, chloretone, and cocaine has been frequently employed in other connections. The sequence of events in this action is suggestive of a distinction between two categories of nervous mechanisms. Whole specimens or fragments were exposed to these agents and tested from time to time for their responses to mechanical stimulation. It was found that local responses of the area immediately about the spot stimulated persisted long after the cessation of more general ones, involving major conduction paths.

The only other drug whose action on the nervous system has been tested is strychnine. Parker (1919), discussing some experiments of Moore (1917), suggests that this drug might afford a physiological test for the presence of synapses. Moore had found that strychnine exerted no effect on certain coelenterates, a slight effect on some echinoderms, and a considerable one on certain crustacea and cephalopods, a series which leads nicely up to the well-known effect on vertebrates generally regarded as a heightening of irritability by decrease of synaptic resistance. Whole specimens of *Saccoglossus pusillus* and pieces of *Balanoglossus occidentalis*, immersed for periods up to several hours in concentrations of from 1 : 1000 to 1 : 100,000 of strychnine sulphate in sea water, showed no effect whatsoever. The responses appeared entirely normal in character, strength, and threshold of stimulation.

This experiment points to a condition similar to that of coelenterates and different from that of higher organisms. But, since the existence of synapses in coelenterates, long denied, has recently been claimed (Bozler, 1927; Hanström, 1928; Woollard and Harpman, 1939), this physiological difference cannot be safely interpreted as proving anatomical continuity. Nor can I follow Parker (1919) and Ariëns Kappers (1929) in assuming that the existence of a physiological nerve net, evidenced by the properties of diffuse transmission, autonomy, etc., necessarily means protoplasmic continuity of neurons. At present it cannot be said whether the physiological nerve net of balanoglossids is synaptic or asynaptic.

Summary

1. A picture of the functional plan of organization of the nervous system of balanoglossids, based on experiments with *Saccoglossus pusillus* and *Balanoglossus occidentalis*, is offered. The state of the receptor and effector mechanisms is also reviewed and correlated with the habit of life.

2. These animals are shown to be highly dependent on a general superficial ciliature whose complex behavior suggests the possibility of nervous control.

3. A great abundance of several types of integumentary glands capable of voluminous secretion, represents a second important effector system. Lacking any physiological evidence of the nature of the products and the variations in activity of the glands, nothing can yet be said regarding the relation of this apparatus to the nervous system.

4. Identification of the organs of light production has not been made. Crozier's argument for their independence from the nervous system is controverted but no positive statement of their nature and control can yet be made.

5. The muscular system is characterized by sluggish action and produces movements of a simple generalized character, lacking differentiation into a variety of reflexes. The paucity of clearly defined and diversified responses greatly limits the possibilities and conditions the nature of experiments on the nervous system.

6. The receptors, likewise, are at a low level of development, lower than that of many coelenterates, being represented by generalized, scattered sense cells. The mechanism of the photic response is discussed in the light of new experiments.

7. The nervous system is shown experimentally to be diffuse and superficial.

8. Properties characteristic of nerve nets—diffuse transmission, decremental conduction, and neuromuscular autonomy of every small fragment of body wall—are demonstrated to exist in balanoglossids. But the presumption of anatomical continuity of neurons from these physiological properties is not assumed, though the facts point to relations resembling those of coelenterates and differing from those of higher animals.

9. The physiological nerve net has been modified correlative with the elongated, bilaterally symmetrical body and the development of great conduction paths in the mid-dorsal and mid-ventral lines. Conduction through the general plexus is more easily induced in the transverse plane than longitudinally.

10. Specialized conduction tracts are demonstrated which correspond to the known histologic concentrations of punctate tissue.

11. Of these, the ventral tract in the trunk is less dispensable than the dorsal, but the latter may be slightly more specialized.

12. Almost no suggestions have been found that these cords, including the internal nerve cord of the collar, function in a ganglionic or central nervous capacity. They are all primarily conduction paths.

13. These facts, together with the general behavior of the animals and the high degree of autonomy of parts are considered to render inappropriate the designation "central nervous system" for the collar nerve cord or any or all of the concentrations of nerve tissue in balanoglossids.

14. The picture of the nervous mechanism of the Enteropneusta is that of an exceedingly primitively organized system. This must be emphasized, but is not considered to affect, one way or the other, the morphologic arguments concerning the relations of these animals and the chordates.

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THE EFFECTS OF VANADIUM, COPPER, MANGANESE AND IRON ON THE SYNTHESIS OF PROTOPLASM BY CHILOMONAS PARAMECIUM [1]

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About forty years ago it was believed that only ten elements are necessary for the synthesis of living matter—carbon, oxygen, hydrogen, nitrogen, phosphorus, sulfur, calcium, magnesium, potassium and iron. It was known before then that many heavy metals besides iron are found in living matter but it has been only during recent years that their indispensability in some organisms and beneficial action in others have been demonstrated. Vanadium, copper, and manganese are among these; however, practically nothing has been done concerning the effect of these elements on the protozoa.

An excellent opportunity to make such a study is offered by the colorless biflagellate protozoan, *Chilomonas paramecium*, which can synthesize starch, fat, proteins and protoplasm from relatively simple compounds under environmental conditions which can be accurately controlled. The results presented in the following pages were obtained in a study of the effects of vanadium, copper, manganese and iron on the rate of reproduction of this organism.

Material and Methods

The original specimens of *Chilomonas paramecium* used in these experiments were obtained from a strain which has been maintained in the Zoölogical Laboratory of The Johns Hopkins University for eight years, at first, in sterile acetate-ammonium solution (Mast and Pace, 1937) and lately in a sterile modified acetate-ammonium solution (Table I). The modified acetate-ammonium solution will be referred to as the acetate-ammonium solution.

Kahlbaum chemicals "highest purity" were used exclusively. All water used in the preparation of solutions was redistilled in a tandem Pyrex glass still (Mast, 1928). Chemically clean Pyrex glass double

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depression slides, Erlenmeyer flasks, and pipettes were used throughout in the experimental work.

Tests were made to ascertain whether the Kahlbaum salts used to prepare the acetate-ammonium solution contained manganese and ferric iron. The test used to detect manganese depends upon the formation of $KMnO_4$ from KIO_4 and salts of manganese when together in acid solution. By this method concentrations of manganese as low as 10^{-5} M can be detected. By using concentrated solutions of salts in which manganese cannot be detected, diluted 1000 times in making the acetate-ammonium solution, it is certain that the concentration of manganese in the culture medium did not exceed 10^{-8} M.

Two tests were used for iron, one based on the formation of $Fe_4[Fe(CN)_6]_3$ (Prussian blue) by ferric iron in the presence of $K_4Fe(CN)_6$ and the other on the formation of $Fe(CNS)_6^{---}$(ferric thiocyanate ion) by ferric iron in the presence of KCNS (Feigl, 1937; pp. 93 and 95). By these methods a concentration of iron as low as 5×10^{-6} M can be detected. By using concentrated solutions of salts in which iron cannot be detected, diluted 1000 times in making the acetate-ammonium solution, it is certain that the concentration of iron in the culture medium did not exceed 5×10^{-8} M. Kahlbaum contends, however, that the $CaCl_2$ used contained 0.0001 per cent iron. If this is true the concentration of iron was at least 1.98×10^{-10} M.

TABLE I

Composition of the modified acetate-ammonium solution used to culture *Chilomonas paramecium*. Hydrogen ion concentration adjusted to pH 6.8 by means of HCl.

Chemicals	Mgm. per 100 cc. Water	Molar Concentrations
$NaC_2H_3O_2$	150.00	0.011
K_2HPO_4	20.00	0.0011
NH_4Cl	46.00	0.0086
$(NH_4)_2SO_4$	10.00	0.00076
$CaCl_2$	1.16	0.00010
$MgCl_2$	1.00	0.00010
HCl	Variable	Variable

The effects of the heavy metals on *Chilomonas* were ascertained by comparing the growth of isolated specimens in acetate-ammonium solution to which varying amounts of compounds of heavy metals were added with the growth of specimens in acetate-ammonium solution to which none were added.

The solutions containing various amounts of heavy metals were prepared as follows: To 49.5 cc. of acetate-ammonium solution in a chemically clean 125 cc. Pyrex glass Erlenmeyer flask enough of the compound of the heavy metal in 0.5 cc. of solution was added to yield

50 cc. of solution containing the highest concentration tested. Then by serial dilution, 50 cc. of each concentration of the metal being tested was prepared in acetate-ammonium solution in individual 125 cc. Erlenmeyer flasks.

The hydrogen ion concentration of these solutions, except in the higher concentrations of the compounds of the heavy metals, was pH 7.2. Since *Chilomonas* grows best at pH 6.8 to 6.6 (Mast and Pace, 1938), enough HCl was added to increase the hydrogen ion concentration to pH 6.8. The amount of HCl needed varied with the kind and concentration of the compound of the heavy metal in the solution. The highest concentration of $VOCl_2$ required none; the highest concentration of Na_3VO_4 required 0.1 cc. 0.4 M; and the other high concentrations and all low concentrations required amounts varying between these. The hydrogen ion concentration was measured daily and maintained at pH 6.8 to 6.6 throughout each experiment.

After the solutions were prepared, the flasks were stoppered with non-absorbent cotton, heated in an oven to 80° C. and kept at this temperature for about 20 minutes. This heating was repeated daily, resulting in solutions which were always entirely free of bacteria and molds.

The experiments were performed as follows: Two dry clean double depression slides were put into each of a number of Petri dishes, heated to 135° C. and left at least 30 minutes. After cooling, each Petri dish was labeled to indicate the kind of solution which was to be put into it, and then 0.1 cc. of that solution put into each of the four depressions. (A different pipette was used for transferring each solution.) One chilomonad was then transferred to each depression in all Petri dishes, directly from a sterile clone which had been growing in acetate-ammonium solution for two or three days. The Petri dishes were then put into an incubator in which the air was very humid and the temperature 24 $\pm$ 0.5° C. After 24 hours the number of divisions that had occurred in each depression was recorded and one individual from each depression was transferred to another depression containing fresh solution of the kind from which it had been taken. The specimens which were not transferred were either discarded or stained to ascertain the amount of starch and fat in them. This procedure was repeated daily as long as the experiment was continued.

Vanadium

Vanadium is usually thought of as a rare metal. This, the evidence shows, is hardly true. It occurs in the crust of the earth to the

extent of 0.017 per cent, whereas copper, zinc, and lead occur respectively to the extent of only 0.0104, 0.0039 and 0.0020 per cent (Marks, 1929).

The occurrence of vanadium in living systems is rare, but it has been known for a long time to occur in plants. According to Suzuki (1903), Lippmann found it in 1888 in the ash of sugar beets, and Demarcay (1900) found it in the wood of oak, elm, poplar and pine trees, and the grape vine.

Henze (1932) found vanadium in 1911 in the blood corpuscles of the ascidian, *Phallusia mammillata*. Vinogradov (1930) found it in *Phallusia obliqua* and remarked that this species is so numerous along the shores of the Russian Arctic Sea that a great accumulation of vanadium now exists there. Vinogradov postulated that the metal functionally takes the place of iron and copper in the blood of the ascidians, implying that it functions in the carrying of oxygen. Henze presented evidence against this view and suggests that the divalent vanadium of the ascidian blood in some way serves as a reducing agent in the formation of the cellulose in the tunic.

Experimental Procedure

The effects of partially reduced vanadium (tetravalent), in $VOCl_2$, and of oxidized vanadium (pentavalent), in Na_3VO_4, were ascertained as follows: Varying concentrations of each were prepared in acetate-ammonium solution, the hydrogen ion concentration adjusted, the solution kept free of bacteria and molds and the experiments made as described above.

Seven experiments were made to ascertain the effect of $VOCl_2$. Of these experiments one was continued 26 days, one 40 days, two 12 days, two 14 days and one 15 days. The chilomonads used in all except one of the experiments were selected from ordinary vigorous cultures. Those used in this experiment were obtained from a culture produced by continuously selecting slow growing specimens for many generations. The former divided considerably more frequently than the latter under identical conditions.

In the former experiments the four original lines in each concentration were continued for the duration of the experiment, but in the latter new lines were started every eight days from a line that had divided regularly throughout the preceding eight days, twice in acetate-ammonium solution containing no vanadium and twice in acetate-ammonium solution containing an optimum concentration of $VOCl_2$. The latter were washed free of vanadium before transferring.

Three experiments were made to ascertain the effect of Na_3VO_4.

Two were continued 15 days and one 12 days. In each the chilomonads used were selected from ordinary vigorous cultures.

The results obtained are presented in Tables II and III and in the following paragraphs.

Results

Table II shows that in experiment I as the concentration of $VOCl_2$

TABLE II

Effect of partially oxidized vanadium (tetravalent), in $VOCl_2$, in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature, 24 ± 0.5° C.

Molar Concentrations of $VOCl_2$	Average Number Divisions per Day of Four Lines	
	Experiment I (26 days)	Experiment II (40 days)
0	3.19±.040*	2.20±.033*
10^{-7}	3.33±.054	2.39±.027
10^{-6}	3.58±.051	2.43±.029
4×10^{-6}	3.58±.064	———
5×10^{-6}	———	2.43±.029
7×10^{-6}	3.38±.063	———
10^{-5}	3.65±.041	2.42±.029
4×10^{-5}	3.67±.039	———
5×10^{-5}	———	2.54±.029
7×10^{-5}	3.61±.054	———
10^{-4}	3.33±.048	2.33±.030
3×10^{-4}	2.97±.086	———

* Probable errors.

increased the rate of reproduction in *Chilomonas* increased from 3.19 divisions per day in acetate-ammonium solution with no vanadium to 3.67 at 4×10^{-5} M and then decreased to 2.97 at 3×10^{-4} M, and that in experiment II the rate of reproduction increased from 2.20 divisions per day in acetate-ammonium solution with no vanadium to 2.54 at 5×10^{-5} M and then decreased to 2.33 at 10^{-4} M. These results show then that partially reduced vanadium (tetravalent), in $VOCl_2$ at certain concentrations, is definitely beneficial for growth in *Chilomonas* and that the optimum concentration is between 10^{-6} M and 7×10^{-5} M.

In some of the experiments which were continued for shorter periods the concentration of $VOCl_2$ was lower than 10^{-7} M and in others it ranged from 10^{-7} M to 10^{-4} M. No verifiable acceleration of growth was obtained in the experiments with concentrations lower than 10^{-7} M, but definite acceleration was obtained at approximately 10^{-6} M in the other experiments.

TABLE III

Effect of oxidized vanadium (pentavalent), in Na_3VO_4, in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature, 24 ± 0.5° C.

Molar Concentrations of Na_3VO_4	Average Number Divisions per Day of Four Lines (15 days)
0	3.76±.063*
10^{-12}	3.74±.057
10^{-10}	3.68±.054
10^{-8}	3.59±.049
10^{-7}	3.73±.045
10^{-6}	3.52±.047
10^{-5}	3.65±.051
10^{-4}	3.63±.052
10^{-3}	3.50±.044

* Probable errors.

Table III shows that there was no increase in rate of reproduction of *Chilomonas* in acetate-ammonium solution containing oxidized vanadium (pentavalent), in Na_3VO_4, but that in the highest concentration tested the rate of reproduction was considerably lower than in the

TABLE IV

Effect of Na_3VO_4 and NaCl in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature 24 ± 0.5° C.

Molar Concentrations of Sodium	Average Number Divisions per Day of Four Lines (7 days)	
	Na_3VO_4	NaCl
0	3.34±.067*	3.34±.067*
3×10^{-3}	3.00±.093	3.28±.060
3×10^{-7}	3.35±.082	3.37±.071

* Probable errors.

acetate-ammonium solution. This experiment was repeated several times. Essentially the same results were obtained in all. Since a molecule of Na_3VO_4 contains three atoms of sodium and only one of vanadium, the question arises whether the inhibition in the rate of growth was due to the sodium or to the vanadium. To answer this question *Chilomonas* was grown in acetate-ammonium solution and in this solution which contained respectively Na_3VO_4 and NaCl in such quantities that the concentration of sodium was the same in both. The results obtained are given in Table IV.

Table IV shows that during seven successive days the average

number of divisions per day in acetate-ammonium solution containing 10^{-3} M Na_3VO_4 was 0.34 of a division less than in the acetate-ammonium solution with no Na_3VO_4 or NaCl, and that in the solution containing 3×10^{-3} M NaCl the average was only 0.06 of a division less. It also shows that the frequency of division in the solution containing 10^{-7} M Na_3VO_4 and in that containing 3×10^{-7} M NaCl was essentially the same as in the acetate-ammonium solution. These results demonstrate therefore that the decrease in the rate of growth in 10^{-3} M Na_3VO_4 was due to the vanadium and not to the sodium in it. This conclusion is supported by the rate of reproduction in 3×10^{-4} M $VOCl_2$ (Table II).

Discussion

The fact that *Chilomonas* can tolerate Na_3VO_4 in concentrations as high as 10^{-3} M indicates that vanadium in this form is not toxic for protozoa. This conclusion is supported by the results obtained by Proescher and Seil (1917). They found that *Opalina ranarum* can tolerate concentrations of $Na_2V_4O_{11}$ as high as 0.284 M.

In other organisms vanadium has been found to be beneficial or detrimental depending on the concentrations. Suzuki (1903) showed that low concentrations of VSO_4 cause increase in the rate of growth of the roots of barley plants but not the total growth and that high concentrations cause cessation of growth. Scharrer and Schropp (Willis, 1936) found that in high concentrations of $NaVO_3 \cdot H_2O$ plant growth is retarded, if not completely stopped, but that in low concentrations there are indications of acceleration, and Shibuya and Saeki (Willis, 1936) report acceleration of plant metabolism by vanadates. Proescher and Seil (1917) report only injurious effects of vanadium on wheat seedlings.

Konishi and Tsuge (1933) studied the elements in soils in which the nitrogen-fixing bacterium *Azotobacter chroococcum* grows. They found by spectroanalysis that vanadium is always present in soil in which *Azotobacter* makes good growth, and that if either VCl_2 or $NaVO_3$ is added in concentrations of either 10^{-4} or 10^{-5} M to an artificial culture medium the amount of nitrogen fixed is five to ten-fold greater than that fixed in culture medium containing no vanadium.

Copper

According to Elvehjem (1935) Meissner first demonstrated, in 1817, that copper occurs in plants. It was not until about a century later that its general occurrence in plants was established by Guerithault (1920), Maquenne and Demoussy (1920) and others. These workers found considerable amounts in fresh plant tissues and seeds. Ma-

quenne and Demoussy found it in so many plants that they suggested that it must be essential in plant growth. Since then numerous investigators (Felix, 1927; Allison, Bryan and Hunter, 1927; Orth, Wickwire and Burge, 1934) have demonstrated that many soils are improved for plant growth by the addition of $CuSO_4$, and Sommer (1931) demonstrated that the element is necessary for plant growth. More recently, Kubowitz (1937) has shown that the polyphenol oxidase of the potato is a copper-containing protein. This is a possible explanation of the necessity of copper for plant growth.

Raulin (1869) believed that probably other metals besides iron and zinc are necessary for the growth of *Aspergillus niger*. Ono (1902) confirmed this belief when he obtained greater dry weights of *Aspergillus niger* by adding small amounts of copper to the culture solution. Lepierre (1913) maintains that zinc in Raulin's solution can be replaced, at least partially, by copper.

Up until recent years only two natural occurrences of copper in animals were known—in hemocyanin and in turacin, the red pigment of the feathers of the Turaco birds of South Africa. Thudichum (1901) claimed that he found it in the human brain, but his claim was not accepted until Bodansky (1921) found considerable amounts in the brains of four people. Now, because Hart, Steenbock, Waddell and Elvehjem (1928) demonstrated that highly purified iron salts are effective in correcting a deficiency of hemoglobin only when small amounts of copper are present, it is recognized that it plays an important rôle in the life of mammals.

Experimental Procedure

The effect of copper in $CuCl_2$ on the growth of *Chilomonas* was tested. Acetate-ammonium solution containing $CuCl_2$ in various concentrations was prepared, the hydrogen ion concentration adjusted and maintained, the solutions sterilized and the experiments made as described above. The results obtained are given in Table V.

Results

By referring to Table V it will be seen that in acetate-ammonium solution containing concentrations of $CuCl_2$ of 10^{-12} M and less, the frequency of division was essentially the same as in acetate-ammonium solution with no copper (3.76 divisions per day) and that in concentrations higher than 10^{-12} M the frequency of division decreased as the concentration of $CuCl_2$ increased, division ceasing entirely at 3×10^{-7} M. In 3×10^{-7} M the chilomonads divided and lived five or six days. As the concentration increased from this the length of life decreased until at 10^{-6} M they lived only a few minutes.

Discussion

The results concerning the toxicity of copper are in accord with those obtained by others. Nägeli (1893) found that copper kills algae in extremely low concentrations. Seybold (1927) found that *Euglena* lives in 10^{-8} M $CuSO_4$ as long as food lasts, but that in 10^{-7} M it dies in less than half an hour.

Chalkley and Voegtlin (1932) found that $CuCl_2$ in a concentration as low as 2×10^{-8} M kills *Amoeba proteus* and that 2×10^{-9} M depresses growth, increases the rate of mortality, and probably decreases the rate at which food in the food vacuoles is digested and assimilated.

TABLE V

Effect of copper in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature $24 \pm 0.5°$ C.

Molar Concentrations $CuCl_2$	Average Number Divisions per Day of Four Lines (15 days)
0	3.76±.046*
10^{-20}	3.70±.056
10^{-18}	3.74±.062
10^{-16}	3.76±.051
10^{-14}	3.76±.051
10^{-12}	3.79±.045
10^{-10}	3.60±.056
10^{-8}	3.42±.072
10^{-7}	3.24±.088
3×10^{-7}	died
5×10^{-7}	"
8×10^{-7}	"
10^{-6}	"

* Probable errors.

Sommer (1931) found that flax, sunflower and tomato plants mature normally when grown in artificial nutrient medium containing copper but do not if it does not contain copper, and Ono (1902), Bortels (1927) and Roberg (1928) showed that small amounts of copper cause an increase in the total growth of *Aspergillus niger*. This indicates that copper is beneficial for growth in many different organisms.

The fact that the rate of reproduction in *Chilomonas* was no higher in any of the concentrations of copper tested than in acetate-ammonium solution without copper indicates that copper is not beneficial for reproduction in this form. The facts that it has been grown for eight years in this laboratory in solutions composed of chemicals of the highest purity with no copper added and that the water in these

solutions was triply distilled in a tandem Pyrex glass still and was kept in Pyrex glass containers, i.e. in glass which according to the makers contains no copper (communication from the Corning Glass works) strongly indicate that copper is not needed for growth in *Chilomonas*.

Manganese and Iron

McHargue (1926) says that in 1788 Scheele showed that pyrolusite (MnO_2) is the oxide of manganese which was then a new metal and that manganese is assimilated by plants. Manganese has since been found in so many plants by Maumené (1884), Headden (1915) and Bertrand and Rosenblatt (1921) that it is now believed to be in all (Miller, 1931).

The addition of manganese to soil was early found to be beneficial to the growth of plants (Nagaoka, 1903; Bertrand, 1905; Brenchley, 1910; McHargue, 1926; and Bryan, 1929). Bertrand (1905) obtained such poor growth in plants with deficiencies of it that he suspected it to be indispensable for growth. This was proved to be true by McHargue (1926), Gilbert and Pember (1931), and McHargue and Calfee (1932).

According to Oettingen (1935) manganese has been found in the blood, the liver and the kidneys in relatively large quantities and in lesser quantities in all other tissues in animals. Its functional rôle in animals is, however, not definitely known. Some investigators (Titus, Cave and Hughes, 1928; Myers and Beard, 1931) found it beneficial in blood regeneration and others (Waddell, Steenboch and Hart, 1929; Orent and McCollum, 1931; and Mitchell and Miller, 1931) found it either non-beneficial or only negligibly beneficial. Orent and McCollum found that rats fed on a diet free of manganese grow to maturity in an apparently normal manner, but that their offspring are undersized and inferior in appearance and that they are not suckled by their mothers. They also found that male rats raised on such a diet develop normally but have degenerated testes.

Gris discovered in 1845 that iron is a constituent of chlorophyll-bearing plants (Bortels, 1927). Since then it has been established that green plants which are deprived of it become chlorotic due to the absence of chlorophyll (Miller, 1931). Raulin (1869) found that if iron is added to culture media in which *Aspergillus niger* is growing the total weight produced is greatly increased.

Iron is found in nearly all if not all animal tissues. It is in all red blood corpuscles and Jones (1920) found it in the nuclei of cells of the liver and kidney and in the cytoplasm and the nuclei of cells of the spleen in adult and foetal guinea pigs. He found it in various tissues of the sparrow, frog, fish, crayfish, oyster, earthworm and

hydra, and says that staining reactions for it are stronger in lower animals than in higher and in foetal mammalian tissues than in adult.

It is well known that iron is closely associated with the transfer of oxygen by blood. In plants it is thought to act catalytically in the formation of chlorophyll (Wolff, 1913). Warburg (1925) contends that in all cells it functions mainly in respiration and that this is due to the catalytic action of a hematin derivative.

Experimental Procedure

The effect of manganese in $MnCl_2$ and iron in $FeCl_3$ on the growth of *Chilomonas* was tested in accord with the preceding experiments on vanadium and copper. The results obtained are presented in Table VI.

TABLE VI

Effect of manganese and iron in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction of *Chilomonas paramecium*. Temperature 24 ± 0.5° C.

$MnCl_2$ and $FeCl_3$ Added; Molar Concentrations	Average Number Divisions per Day of Four Lines	
	$MnCl_2$ (19 days)	$FeCl_3$ (17 days)
0	3.28±.055*	3.30±.045*
10^{-9}	3.25±.045	3.38±.046
5×10^{-9}	3.17±.063	3.37±.045
10^{-8}	3.31±.058	3.37±.041
5×10^{-8}	3.39±.049	3.40±.045
10^{-7}	3.33±.048	3.33±.049
5×10^{-7}	3.40±.046	3.39±.042
10^{-6}	3.25±.041	3.33±.057
5×10^{-6}	3.28±.051	3.47±.045
10^{-5}	3.38±.042	not tested
5×10^{-5}	3.28±.049	" "
10^{-4}	3.39±.041	" "

* Probable errors.

Results

Table VI shows that in the solutions to which $MnCl_2$ was added the frequency of division varied from 3.17 to 3.40 per day and that in those to which $FeCl_3$ was added it varied from 3.33 to 3.47 per day, but that there is no indication of any correlation between the concentration of either manganese or iron and the frequency of division and no statistically significant difference between the rate of reproduction in the solutions to which iron and manganese were added and the acetate-ammonium solution. These tests were repeated and essentially the same results were obtained. They therefore show that iron and manganese neither augment nor retard growth in *Chilomonas*.

Cultures of *Chilomonas* have been maintained in this laboratory in acetate-ammonium solution for nearly eight years without any indication of loss of vigor. Neither iron nor manganese was added to the solution. It consequently appears that neither is necessary for growth in *Chilomonas*. Kahlbaum maintains, however, that the $CaCl_2$ used in the solution contains 0.0001 per cent iron and some of the salts used may have contained a trace of manganese. The fact that *Chilomonas* grows in acetate-ammonium solution does not therefore prove that iron and manganese are unnecessary, but the fact that neither was detected in the tests described above which are sensitive to 5×10^{-8} M for iron and 10^{-8} M for manganese demonstrates conclusively that if these metals are necessary extremely minute quantities suffice.

Discussion

Molisch (1894) grew *Aspergillus niger* and *Penicillium* sp. in a culture medium to which no iron was added. However, analysis showed that they contained iron, and when iron was added to the culture medium the amount of growth was much greater. Molisch therefore concluded that these two molds need iron. The conclusion that *Aspergillus* needs iron has been amply verified by Sauton (1911), Steinberg (1935), Bortels (1927) and Roberg (1928). Sauton also found that *Penicillium* needs iron, but that the molds *Mucor mucedo*, *Rhizopus nigricans* and *Racoelium coellare* do not need it.

Bertrand and Javillier (1911) found that the dry weight of *Aspergillus niger* increased as the manganese content increased to 1000 mg. per 100 cc. and then decreased. Later Bertrand (1912) obtained augmentation of a crop of mold by the addition of an amount of manganese equivalent to one mg. per 10,000 liters of nutrient medium and because of this evidence suggested a catalytic rôle of the metal.

Hotchkiss (1923) found that iron does not accelerate the growth of *Bacterium coli*, although it has been found to accelerate the growth of many bacteria. Koser, Finkle, Dorfman and Saunders (1938) tested the possibility that inorganic salts present as impurities in preparations of spleen, liver, and yeast cause the growth-promoting activity of these preparations. They found that salts of neither iron, manganese nor copper were responsible for the growth in five bacteria and a yeast caused by additions of the preparations; however, they point out that their results do not unequivocally invalidate the evidence of the numerous investigators who have found that small amounts of the heavy metals do accelerate cell reproduction.

Hall (1937) maintains that manganese causes acceleration in growth in *Euglena anabaena*, but not in *Colpidium campylum* and *Astasia*.

These studies show that, although the heavy metals cause acceleration of growth in many organisms, they do not do so in all, and they seem to be indispensable to some forms and not to others. As already stated, the results of Tables V and VI show that the growth of *Chilomonas* is not accelerated by additions of copper, manganese and iron, and they indicate that these metals are not indispensable to *Chilomonas*. More evidence, however, is highly desirable.

Some evidence concerning the necessity of iron is found in the results obtained in the study of respiration in *Chilomonas*. If *Chilomonas* respires by means of an enzymatic iron-porphyrin system, the most common of which is that involving cytochrome and indophenol oxidase, iron is obviously necessary. Cytochrome, a hemochromogen, and indophenol oxidase are found in most aerobic organisms and tissues, including mammalian tissues, plants, insects, yeasts and aerobic bacteria (Meldrum, 1934). However, tests of a heavy suspension of chilomonads by means of a hand spectroscope and a microspectro-

FIG. 1. Camera outlines showing the variation in starch and fat content in specimens of *Chilomonas paramecium* in acetate-ammonium solution (Table I) and in this solution with vanadium (5×10^{-4}M), copper (10^{-10}M), manganese (5×10^{-6}M) and iron (10^{-6}M) added respectively. ○, starch; ●, fat. Note that the starch and fat content varied from no starch and much fat to much starch and little fat and that this occurred in specimens grown in acetate-ammonium solution as well as in this solution with metals added.

scope revealed no absorption bands characteristic of cytochrome, and Hutchens (1939), working in this laboratory, reports that the respiratory system of *Chilomonas* is not sensitive to cyanide, which indicates the absence of indophenol oxidase and cytochrome.[2]

Relation between Vanadium, Copper, Manganese and Iron and the Amount of Starch and Fat in Chilomonas

From time to time chilomonads grown in acetate-ammonium solution and in this solution containing the various metals under consideration were studied under the microscope to ascertain the effect of the metals on the starch and fat content. This was done as follows:

[2] Since this paper went to press, Hutchens, by personal communication, has reported the presence of cytochrome and a sensitivity of respiration to cyanide in *Chilomonas* when grown in a more complex medium in which greater numbers of organisms are obtained.

Equal numbers of specimens were selected at random from each of the four lines in a given solution and put into a vaseline ring on a glass slide with as little water as possible; then lugol solution was added and a few minutes later sudan III. This fixed the chilomonads and stained the starch and fat in them. Specimens representing the extent of variation in fat and starch content were then selected and camera outlines made. One of the specimens in each solution which contained the greatest amount of starch and one which contained the greatest amount of fat are presented in Fig. 1.

This figure shows that the relative content of starch and fat varied enormously in different specimens, but that it varied just as much in those grown in acetate-ammonium solution as in those grown in this solution containing any one of the metals used and that there is no correlation between this variation and the kind or the concentration of these metals.

Observations on the relation between the rate of reproduction of the chilomonads in these solutions and the starch and fat content show that the fat content varied inversely and the starch content directly with the rate of reproduction in all of the solutions, the acetate-ammonium solution as well as this solution plus the metals. The relative amount of starch and fat in *Chilomonas* is therefore correlated with the rate of reproduction.

Summary

1. Experiments were performed to ascertain the effect of vanadium ($VOCl_2$ and Na_3VO_4), copper ($CuCl_2$), manganese ($MnCl_2$) and iron ($FeCl_3$) in acetate-ammonium solution on the rate of reproduction and the synthesis of starch and fat in *Chilomonas paramecium*.

2. Tetravalent vanadium in $VOCl_2$ causes marked increases in the frequency of division in *Chilomonas* which vary with the concentration. As the concentration of $VOCl_2$ added to the acetate-ammonium solution was increased, the frequency of division increased to a maximum at approximately 10^{-5} M and then decreased. Pentavalent vanadium in Na_3VO_4 causes no increase in frequency of division. Tetravalent vanadium at certain concentrations is therefore beneficial for growth of *Chilomonas* and pentavalent vanadium is not.

3. Neither copper, manganese nor iron in acetate-ammonium solution causes any statistically significant increase in the frequency of division of *Chilomonas*, and neither manganese nor iron causes a significant decrease in the frequency of division, but as the concentration of copper increases from 10^{-12} M the frequency of division decreases, ceasing entirely at 3×10^{-7} M.

4. *Chilomonas* has been grown continuously for eight years in acetate-ammonium solution with no additions of copper, manganese or iron. This shows conclusively that if the metals are needed extremely minute quantities suffice, but it does not prove that they are unnecessary for there are doubtlessly traces of them in the solution.

5. The starch and fat content of *Chilomonas* varied from no starch and much fat to much starch and little fat in all the solutions used; therefore, this variation was not caused by the addition of either vanadium, copper, manganese or iron. It was found in all solutions to be correlated with the rate of reproduction.

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STUDIES ON THE TREMATODES OF WOODS HOLE. III. THE LIFE CYCLE OF MONORCHEIDES CUMINGIAE (MARTIN) WITH SPECIAL REFERENCE TO ITS EFFECT ON THE INVERTEBRATE HOST

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This work was done during the several past summers spent at the Marine Biological Laboratory. A synopsis of this research was given before the 1939 Columbus, Ohio meeting of the American Society of Parasitologists. *Monorcheides cumingiae* parasitizes the intestines of flounders and eels, two important food fishes, and so may be of some economic importance. Very little work, particularly of an experimental nature, has been done on the life cycles of the members of the family *Monorchidae* to which this species belongs.

The family name *Monorchidae* was erected by Odhner (1911) with *Monorchis monorchis* (Stossich) as the type genus and species. As the family name indicates, one testis is characteristic of the group, but the genus *Monorcheides* is an exception in that two testes are present. The genus *Monorcheides* was created by Odhner (1905) for some small worms found in the intestine of the marine fish, *Lumprenus medius*, collected along the west coast of Spitzbergen.

Although the members of this family are rather widely distributed, very little work has been done to trace the biological relationships of the stages in the life cycles. According to Yamaguti (1938), Nagano (1930) found that a cercariaeum that developed in rediae and encysted in *Bulimus striatus japonicus* (Pilsbury) became the adult of *Asymphylodora tincae*. Yamaguti (1934) states that the species Nagano was working with was probably *A. macrostoma* Ozaki but in a later paper (1938) refers this form to *A. japonica* Yamaguti. Yamaguti (1934) reported finding what he believed to be the larval form of *A. macrostoma* Ozaki encysted in the peribuccal connective tissue of *Chaenogobius macrostomus*, although no feeding experiments were conducted. In 1938 this same author reports that on the basis of anatomical and ecological evidence a cercariaeum and its cyst found in *Bulimus striatus japonicus* (Pilsbury) probably represent stages in the life cycle of *Asymphylodora japonica* Yamaguti. In the same paper he

reported finding the larva of *Asymphylodora macrostoma* Ozaki encysted in the peribuccal connective tissue and gill arches of *Gnathopogon elongatus caerulescens* (Sauvage) from Lake Biwa and from *Cobitus biwae* Jordan and Snyder from the Katura River, Japan. During the past summer it was possible to demonstrate that *Cercaria cumingiae* Martin (1938), when fed under experimental conditions to flounders and eels, develops into an adult belonging to the genus *Monorcheides*.

In addition to the genus *Monorcheides*, the family *Monorchidae* includes species belonging to the following genera which have been collected from widely scattered regions: *Asymphylodora* Looss 1899 from Egyptian (Looss, 1899), Japanese (Yamaguti, 1938), and Indian (Srivastava, 1939) waters; *Bivesicula* Yamaguti 1934 from Japan; *Genolopa* Linton 1910 from Bermuda and Dry Tortugas (Linton, 1910), Beaufort, North Carolina (Manter, 1931), and North Queensland (Nicoll, 1915); *Hurleytrema* Srivastava 1939 from India; *Lasiotocus* Looss in manuscript and *Pristisomum* Looss in manuscript (Odhner, 1911) from Mediterranean and British (Nicoll, 1915) waters; *Monorchis* Looss 1902 from the Mediterranean Sea and British waters (Nicoll, 1915); *Paramonorcheides* Yamaguti 1938 from Japan and from the Galapagos Islands (Manter, 1940); *Paraproctotrema* Yamaguti 1934 from Japan; *Physochoerus* Poche 1925 from the Mediterranean Sea; *Proctotrema* Odhner 1911 from the Scandinavian arctic (Odhner, 1911), from Beaufort, North Carolina, Costa Rica, and the Galapagos Islands (Manter, 1931, 1940), from Japan (Yamaguti, 1934) and from the Karachi and Arabian Seas (Srivastava, 1939); *Proctotrematoides* Yamaguti 1938 from Japan; and *Telolecithus* Lloyd and Guberlet 1932 from Puget Sound and from Panama (Manter, 1940).

Materials and Methods

Living material, preserved whole mounts, and serial sections were used in this study. Living material is particularly useful in working out the finer details of the anatomy of the larval stages. Neutral red was used to stain the living material, while on preserved specimens Mayer's paracarmine was used on whole mounts and Delafield's hematoxylin and eosin were used on sectioned material. The mounting medium, "Clarite," was used and proved very satisfactory.

The mollusks, *Cumingia tellinoides*, were placed in finger bowls and daily observations were made to detect the presence of emerging cercariae. Infected clams were isolated individually and heavy infections of metacercariae were established in them. Heavy infections were produced also in clams that were not infected with sporocysts. In other words, some clams that had not served as first inter-

mediate hosts were used as second intermediate hosts. Infected clams were fed to flounders and eels that had been kept in aquaria for several weeks to allow the maturation of any worms that might have been present in the intestines of these fishes when they were brought into the laboratory.

Observations

The life cycle of *Monorcheides cumingiae* involves the development of sporocysts, cercariae, and metacercariae in the marine bivalve, *Cumingia tellinoides* (Conrad), and the development of the adult, under experimental conditions, in the intestines of eels and flounders.

The Life Cycle

The Sporocyst

The sporocysts are simple sac-shaped structures that develop in the visceral mass of the clam. Because of the large number of sporocysts within a single clam it seems probable that there is a daughter sporocyst generation although this has never been observed. The entire visceral region may be riddled with sporocysts, resulting in partial or complete castration and other disturbances. The intestine was usually surrounded by sporocysts and it is likely that this is the avenue of entrance of the miracidia. The fact that the eggs of this parasite failed to develop when kept for over three weeks in sea water suggests that they must be eaten by the clam in order for development to take place. The wall of the sporocyst is very thin and contains scattered nuclei. However, at the ends of the sporocysts thickened knobs of cells are present and they seem to take part in the formation of germ balls. The nuclei in these knobs enter protoplasmic extensions into the lumen of the sporocyst and, finally, actually leave the cytoplasm behind and take part in cercarial formation. The lumina of the sporocysts contain fragments, probably cellular, which stain with eosin.

The Cercaria

The cercaria bears two conspicuous eyespots in the anterior half of the body which still are evident in the recently encysted metacercaria (Fig. 5). The oral and ventral suckers are approximately equal in size. The excretory system is relatively simple and is represented by the formula $2[(2 + 2) + (2 + 2)]$. A mass of cells directly behind the ventral sucker represents the anlage of the reproductive system. The cercariae emerge from the clam by way of the excurrent siphon and swim about immediately. The light intensity is a controlling factor of the direction of swimming because the cercariae show a definite

negative response to light. Eventually the cercariae come in contact with the tissues of the clam, either by being forcibly taken in by the incurrent siphon or by coming in contact with the mantle or foot through their own swimming efforts. In both cases they soon shed their tails and work their way into the clam tissue where they encyst. Apparently, encystment does not take place prior to a period of swimming, i.e. the cercariae do not encyst in the time interval between emerging from the sporocysts and leaving the clam. The factor, or factors, controlling the time of encystment are unknown. The fact that a swimming period must occur before encystment takes place is of definite advantage to the parasite in increasing its range, since clams other than the infected one may become second intermediate hosts. In this connection it was found that another small clam, *Tellina tenera* Verrill, could serve as a second intermediate host. This same species may very rarely serve as the first intermediate host since one of several hundred examined was infected with sporocysts.

In the original description of *Cercaria cumingiae* Martin 1938, it was postulated that this species probably belonged to the family *Allocreadiidae* because of anatomical similarities to some of the cercariae of this family. However, the experimental results have disproved this postulation. Nevertheless, these similarities suggest a possible relationship of the family *Monorchidae* to the family *Allocreadiidae*.

EXPLANATION OF PLATES

Abbreviations used:

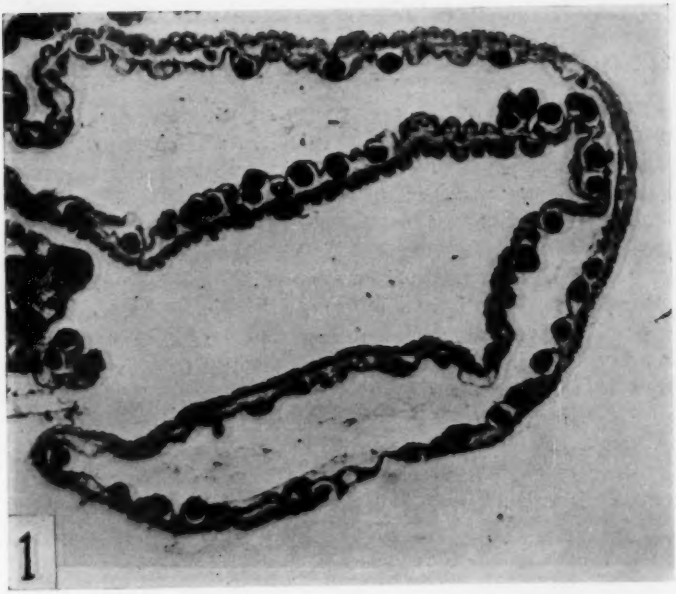
C.....cirrus	*OV*.....ovary
CS.....cirrus sac	*P*.....pharynx
E.....eyespot	*PR*.....prostate tissue
EB.....excretory bladder	*S*.....seminal vesicle
EG.....egg	*SP*.....spine
G.....genital pore	*T*.....testes
I.....intestine	*U*.....uterus
M.....metacercariae	*V*.....vagina
O.....oral sucker	*VI*.....vitellaria

Description of Plate I[1]

Fig. 1. Longitudinal section through the siphon of *Cumingia tellinoides* showing metacercariae just inside the muscular layer of the siphon.

Fig. 2. Section through the gill and part of the body of *Cumingia tellinoides* showing metacercariae in the gill and sporocysts (a) in the body.

[1] I wish to express my appreciation to Dr. E. J. Kohl of Purdue University for making the photomicrographs in Plates I and II.



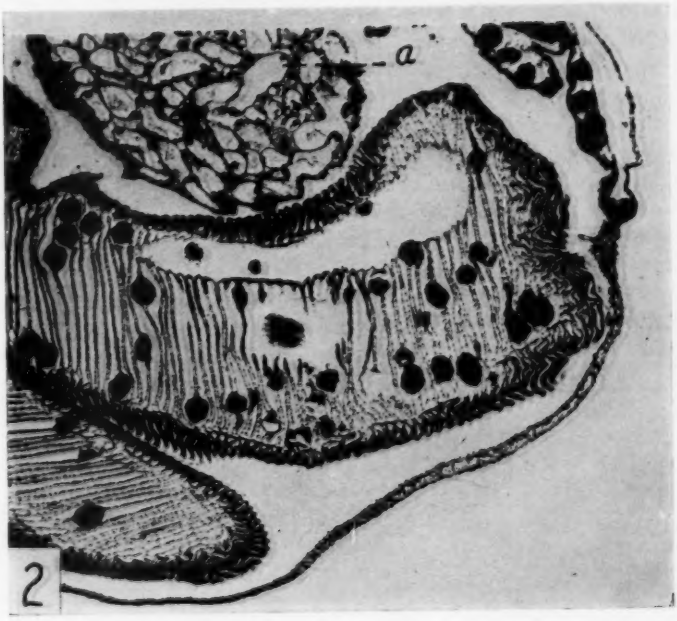


PLATE I

The Metacercaria

Upon shedding its tail and encysting the cercaria becomes a metacercaria. During the metacercarial stage certain important changes occur: the eyespots start to disintegrate, the digestive system becomes much better developed with the ceca reaching to near the posterior end of the body, there may be a slight increase in the number of cephalic glands, the excretory bladder loses its thick-walled appearance and becomes a thin sac due to the breaking down of the cells of the wall, there is an increase of two or three times in the size of the body, and the cuticula increases in thickness. A very interesting change occurs in the staining reaction of the nuclei with the aging of the metacercariae. The nuclei of recently encysted metacercariae can hardly be distinguished but as the metacercariae become older the nuclei stain heavily with Delafield's hematoxylin (Fig. 5). The significance of this change is unknown. During the metacercarial stage there is very little differentiation of the genital region.

The Adult (Figs. 6 and 7)

Under natural conditions, it seems probable that the definitive fish host obtains its infection by eating the siphons or the entire clam infected with metacercariae. *Cumingia tellinoides* and *Tellina tenera* extend their siphons a considerable distance beyond the edge of the shell and these structures could attract the attention of fishes. In the laboratory, small puffers, *Sphaeroides maculatus* (Schneider), were observed to bite off the siphons of *Cumingia* and other clams. In tracing this life cycle, entire infected *Cumingia* were removed from their shells and fed to three flounders and to three eels. Six of each species of fish were reserved as controls. With the exception of the infected *Cumingia*, both experimental and control fishes received the same type of food, namely clam meat, *Venus mercenaria*, *Mya arenaria*, *Modiolus modiolus*, and *Mytilus edulis*, which was examined carefully for any possible trematode infection. One of the eels, however, was

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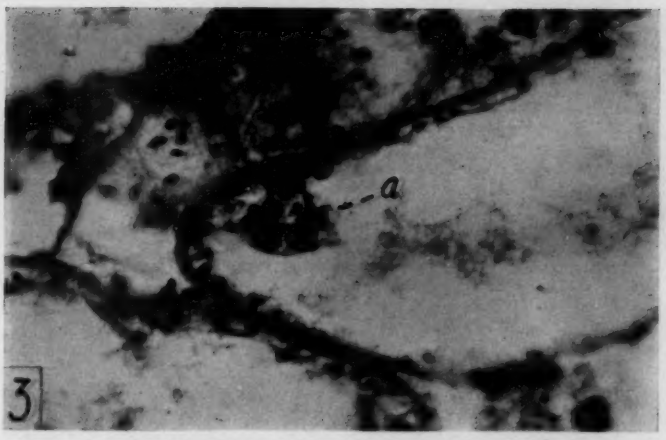
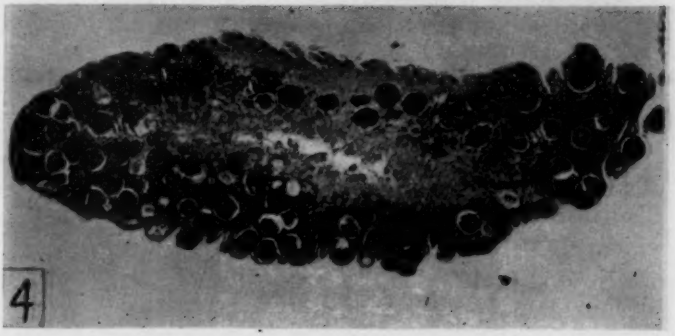
DESCRIPTION OF PLATE II[1]

FIG. 3. Section through a sporocyst showing the enlarged knob (*a*) of cells from which nuclei proliferate to form germ balls which become cercariae.

FIG. 4. Section through the foot of *Cumingia tellinoides* showing the metacercariae and the darkly staining material deposited around them by the clam.

FIG. 5. Section showing a recently encysted metacercaria (*a*) with conspicuous eyespots but showing no nuclei in contrast to older, adjacent metacercariae whose nuclei stain heavily.





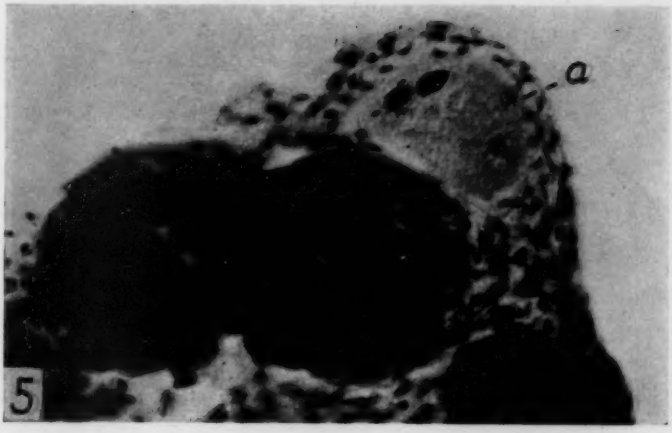
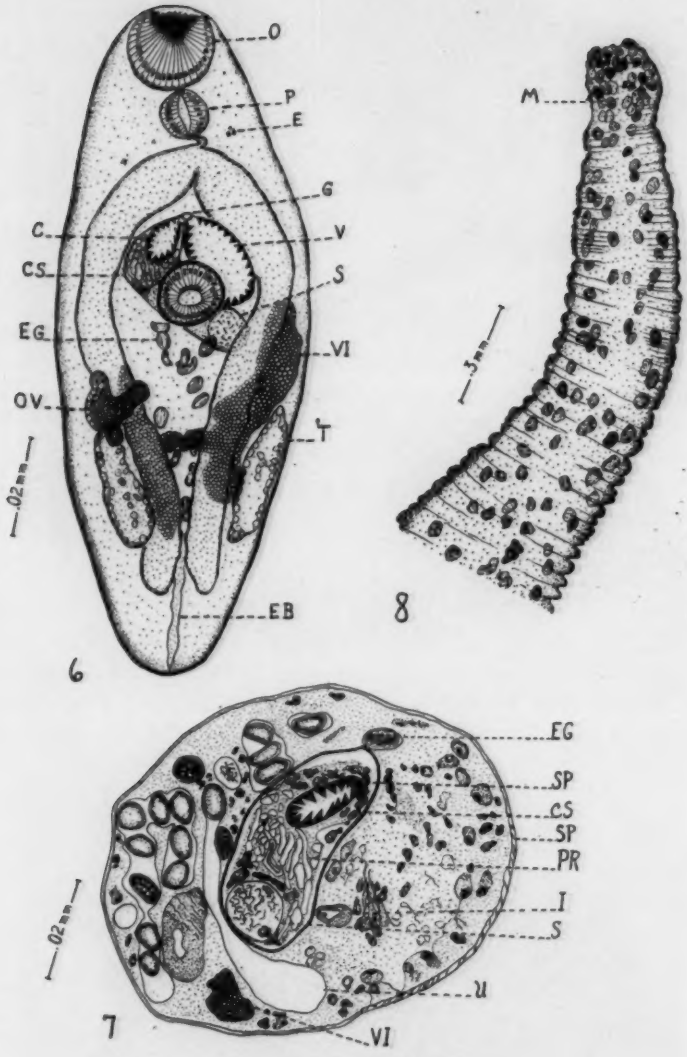


PLATE II



DESCRIPTION OF PLATE III

FIG. 6. Adult *Monorcheides cumingiae.*

FIG. 7. Transverse section of an adult *Monorcheides cumingiae.*

FIG. 8. Whole mount of a siphon of *Cumingia tellinoides* showing the distribution of metacercariae.

fed only infected *Cumingia* and after four weeks of feeding it was found to have an infection of over one hundred *Monorcheides cumingiae* in various stages of development. In all, four of the six fishes experimentally fed infected *Cumingia* were found to harbor *Monorcheides cumingiae* when examined four weeks after the initial feeding. In both the eels and the flounders, the parasites were found in the mucus of the anterior portion of the intestine. No ulceration of the intestine was observed. The controls were negative.

The adults are extremely small trematodes as shown by the following measurements based on five specimens, all of which contained eggs. The body length is 0.255–0.318, with an average of 0.30 mm.; the body width is 0.1–0.164, with an average of 0.142 mm. The cuticula is spined with the spines being somewhat more conspicuous on the anterior half of the body. The oral sucker is terminal or subterminal and measures from 0.0315–0.0415, with an average of 0.0367 mm. in length by 0.0348–0.0498, with an average of 0.0408 mm. in width. The ventral sucker measures from 0.0232–0.0332, with an average of 0.026 mm. in length by from 0.0291–0.0332, with an average of 0.032 mm. in width. The very short prepharynx (about 0.003–0.004 mm. in length) is followed by a pharynx that is on the average 0.0185 mm. in length by 0.022 mm. in width. The esophagus is approximately 0.0124 mm. long and it leads to the forking of the intestine which occurs about midway between the two suckers. The intestinal rami approach, but do not extend to, the posterior end of the body. The reproductive systems are well developed and both the cirrus and vagina are armed with relatively large triangular spines. The genital pore is located in the mid-ventral region just posterior to the forking of the intestine. The cirrus sac is elongate and extends from the genital pore to a short distance posterior to the ventral sucker. It encloses a seminal vesicle, numerous "prostate" cells, and a spined cirrus. The two testes are laterally situated about half-way between the ventral sucker and the posterior end of the body. They are composed of loosely arranged tissue. The single ovary is located just above the testis on the right side of the body. Its margin is irregularly lobate. No seminal receptacle was observed. The coils of the uterus may fill the posterior half of the body, particularly in the region between the testes. The uterus terminates anteriorly at approximately the middle of the median surface of the vagina. The vagina is relatively thick-walled, and is spined throughout its entire length. It is quite large, measuring approximately 0.0224 mm. wide by 0.0332 mm. long. It generally lies on the left side of the body. The spines arming the vagina are similar in shape and size to those found in the cirrus. The

eggs are amber-colored, oval in outline, operculate, and measure about 0.016 mm. long by 0.011 mm. wide. Mature eggs were removed from the uteri of several worms and were kept for over three weeks under conditions favorable for hatching but very little change took place in them. This suggests that the eggs must be eaten by the clam host before development will proceed.

The adult *Monorcheides cumingiae* differs from the other members of the genus in the following ways. The size of the body, suckers, pharynx, and eggs is smaller in *M. cumingiae* than in any other member of the genus. The shape of the vaginal and cirrus spines is triangular in *M. cumingiae* but in the other species, judging from figures, they are much more elongate. The spines of the cirrus of *M. cumingiae* are uniform in size while in *M. diplorchis* Odhner the spines on the median side are longer than those found on the lateral side of the cirrus. The vitellaria of *M. soldatovi* Issaitschikow extend from the posterior border of the pharynx to the level of the middle of the ovary, in *M. diplorchis* the yolk glands extend from the level of the forking of the intestine to a short distance posterior to the anterior margin of the ovary, while in *M. cumingiae* these glands may extend from the posterior margin of the ventral sucker to the anterior margin of the testes. *Monorcheides* (?) *petrowi* Layman probably does not belong to this genus at all because the ovary is posterior instead of anterior to the testes. The genus *Paramonorcheides* Yamaguti is closely related, if not synonymous, with the genus *Monorcheides*. The principal difference between the two seems to be a sac-shaped excretory bladder in the former and a Y-shaped one in the latter. Some specimens of *Monorcheides cumingiae* give the appearance of having a Y-shaped bladder due to the distention of the lower portions of the main collecting ducts. It is likely that Odhner believed that these enlarged collecting ducts were continuations and part of the bladder.

Nagano (1930), according to Yamaguti (1938), has shown that the larval form of *Asymphylodora tincae* is a tailless cercaria, or the so-called cercariaeum, lacking eyespots. It is apparent, therefore, that there are at least two larval types in the family. The larval form of the genus *Bivesicula* Yamaguti 1934 is evidently similar to the cercaria of *Monorcheides cumingiae* in the possession of eyespots since remnants of these structures are found in the adults.

Effect of Parasite on Clam

The effects of the parasite on the clam may be divided into two categories, first the damage done by the sporocysts when the clam serves as the first intermediate host, and second the injury caused by

the cercariae when they re-enter to become metacercariae or when the clam is serving as the second intermediate host. Of the two, the first undoubtedly is more extensive since most of the visceral region may become filled with sporocysts. The sporocysts seem to be more numerous along the wall of the gut and along the periphery of the body in lighter infections but in severe infections they fill the tissues between these two regions. The fact that the sporocysts first appear along the wall of the gut seems to support the evidence obtained from the unsuccessful efforts to hatch the eggs of this worm, that the eggs are eaten by the clam and hatch in the gut, infection taking place through the wall of the digestive tract. The normal tissue seems to be pushed aside and crowded upon itself until disintegration takes place. Here and there between sporocysts portions of host tissue may remain in an apparently normal condition. Just how this replacement of host tissue by the parasite occurs is unknown. Perhaps some of it is by an enzymatic dissolution of the host cells or perhaps it is by the compression of the host tissue by the parasite. If the latter is true, and there is some evidence to support this contention because here and there between the sporocysts distorted host cells can be found, the parasite tissue must be able to exert a greater growth pressure than the host tissue. The fact that host tissue may remain in certain regions entirely surrounded by sporocysts seems to indicate that the action is not an enzymatic one, or if it is, some of the host's cells are more resistant than others to this action.

The effects of the penetration of the cercariae are confined for the most part to those tissues of the clam that are in contact with the water, such as the siphons, mantle, foot, and gills. The siphon tissue, particularly that region next to the lumen and just below the muscular layer, is a favorite one for the penetration of the cercariae (see Fig. 1). Eighty metacercariae were counted in one longitudinal section of a siphon. The distal end of the siphon may become so filled with metacercariae that it presents a frayed appearance. Frequently the heavily infected siphon is autotomized. Although the incurrent siphon generally contains a larger number of metacercariae, the excurrent siphon may become rather heavily infected. Some encystment takes place in the mantle but probably because of its thinness it is incapable of supporting many metacercariae. The muscle of the foot of the clam becomes very heavily infected. Between ninety and one hundred metacercariae were observed in one longitudinal section of the foot. The metacercariae in this region seem to induce the formation of a deeply staining (with Delafield's hematoxylin) deposit which may be an attempt by the clam to protect itself. It, however, is not

a very effective protective device because recently encysted metacercariae were found in tissue that also contained older metacercariae. The mantle also may form small amounts of this deposit, in some cases even when there are no metacercariae present in that particular region. Therefore, it is likely that the deposition of this material is not a specific response to the action of the parasite but, nevertheless, the amount of the material deposited may be increased by the presence of the metacercariae. With the relatively large amount of surface exposed it seems rather remarkable that so few metacercariae are found in the visceral region. Perhaps the presence of sporocyst tissue may act as a barrier to the entrance of cercariae, or, it may be that the visceral tissue does not have sufficient rigidity for the penetration of cercariae for metacercariae are found in regions of the body where muscle fibers occur near the surface. The cercariae frequently penetrate the gill filaments forcing the thin membranes apart so that they surround the metacercariae. Some of the more heavily infected filaments are distorted considerably.

The damage done by the parasite to the invertebrate host is undoubtedly much more extensive than that received by the vertebrate host. However, in spite of the very extensive damage suffered by the clams they lived for several weeks, even without food except for the small amount that would be obtained from the daily changes of sea water.

Summary

1. The life cycle of *Monorcheides cumingiae* (Martin) involves the development of sporocysts, cercariae, and metacercariae in the marine bivalve, *Cumingia tellinoides*, and the development of the adult, under experimental conditions, in the flounder and eel.

2. Another clam, *Tellina tenera*, can serve as an alternative second intermediate host and on one occasion was found to act in the capacity of a first intermediate host.

3. Attempts at hatching the eggs of this trematode failed. Therefore it seems probable that the eggs must be eaten by the clam before further development will take place.

4. In spite of the extremely heavy infections, the clams survived for several weeks with no more food than would be obtained from the daily changes of sea water.

5. A free-swimming period is apparently necessary before the cercariae will encyst. This is an advantage to the parasite in increasing its range.

6. The cercariae encyst in large numbers in the siphons, particularly the incurrent, and foot. Metacercariae are found in smaller numbers in the gills, mantle, and, very rarely, in the visceral region.

7. There is a marked change in the staining reaction of the nuclei of recently encysted and older metacercariae. When both receive the same treatment, the nuclei of the young metacercariae take up little or no Delafield's hematoxylin while the nuclei of the older metacercariae are heavily stained.

8. Although the metacercariae seem to induce the surrounding clam tissues, particularly in the foot, to increase the deposition of a darkly staining material, this substance does not inhibit super infection.

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STUDIES ON THE EFFECTS OF EYESTALK REMOVAL UPON YOUNG CRAYFISH (CAMBARUS CLARKII GIRARD)

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In recent years it has come to be recognized as a result of the work of Megušar (1912), Abramowitz and Abramowitz (1938), Brown and Cunningham (1939), Hanström (1939), and Abramowitz and Abramowitz (1940) that removal of both eyestalks in decapod crustaceans causes the next moult to occur sooner than it would normally. Removal of eyestalks is eventually fatal, as Brown (1938) and Brown and Cunningham (1939) have shown for *Cambarus*, and Abramowitz and Abramowitz (1940) have shown for *Uca*. Brown and Cunningham give evidence, derived from sinusgland implantation, that the factor or factors related to moulting and viability originate in the sinusgland of the eyestalk.

In the above work the general method has been to operate on a number of animals at once, and then to compare the incidence of moulting in this group with that in an untreated group. This procedure gives no information as to the actual amount of shortening of the intermoult following operations, nor has sufficient evidence been presented to prove that the acceleration of moulting is a real shortening of the intermoult and not merely a speeding up of the intermoult in its later stages by the shock of eyestalk removal. The experiments to be described below were intended to clarify these questions by means of operations made after observed moults on animals whose history was known and whose moulting cycles could be followed individually.

To Dr. H. W. Rand, who kindly provided facilities for, and advice during, the preliminary experiments, and to Dr. J. H. Welsh, whose coöperation and criticisms have been invaluable, I wish to express my gratitude.

EXPERIMENTAL

In experiments carried out in 1938, young *Cambarus clarkii* were used, ranging in length from 17 to 24 mm. (rostrum to telson). These were kept in individual dishes at 9–14° C. and fed very lightly. Operations were performed two days after animals were observed to have moulted. Eyestalks were occluded by ligating them at the base with

fine hairs, while as a control operation antennae were similarly ligated and then removed with scissors. Other animals were left intact as controls.

It was found that removal of both eyestalks was always fatal. In the course of the eyestalk ligations discussed here, and some performed in another connection, 60 animals had both eyestalks tied off. Of these, 53 had died by the close of the experiment. Survival ranged from 1 to 38 days following the operation, averaging 17.1 ± 1.14[1] days. If deaths within 10 days are disregarded the average survival was 19.7 ± 1.03 days. That this mortality is not due merely to operative injury is shown by the fact that only 2 deaths resulted from antennal removal among 30 animals.

Eyestalkless animals often moulted before dying, with intermoults shorter than in normal animals, but antennal removal had no significant effect upon the length of the following intermoult. Thirty-four crayfish had their eyestalks ligatured after the first observed moult. All died, but 14 moulted before dying, with intermoults averaging 15.9 ± 1.02 days, while in 29 normal animals the average was 28.9 ±1.65 days, and in 30 animals with antennae removed the average was 31.2 ± 2.28 days. This may indicate a significant shortening of intermoult by eyestalk removal, although the results are unsatisfactory because of the high percentage of deaths in the eyestalkless group.

Accordingly, the work was repeated in 1939, using a modified procedure. The essential feature of this second experiment was that operations were made only on animals in which one or more complete intermoults had been observed. Thus an intermoult following an operation could be compared with the preceding "normal" intermoult in the same animal. To hasten growth and moulting the animals were kept at room temperature and were fed liberally, the effort being made to supply as much food as they could eat. This had a marked effect upon survival that will be mentioned below.

Eyestalks were removed, with the animals held on ice, by pinching them off at the base with watchmaker's forceps. As a control operation the severe procedure of cutting off the retinal portion of the eyestalk with fine scissors was used. This caused an open wound with much bleeding. It was found advisable to perform the operations of eyestalk or retinal removal in two steps, taking off the second eyestalk or retina 12 hours after the first. Cautery was not employed.

[1] In this and following cases where standard errors are given, the errors are standard error of the mean, given by the formula $\frac{\text{standard deviation}}{\sqrt{\text{no. of variates}}}$.

The 122 *Cambarus clarkii* used ranged in length from 8 to 12 mm. (rostrum to telson) at the start of the 7-week observation period. Observations were made twice daily. When an animal had moulted twice (one intermoult), it was assigned to one of three experimental groups. The first animal was left as an intact control, the second had both eyestalks pinched off, the third had both retinas cut off. This series of assignments was repeated as moults occurred among the animals. Operations were started 24 hours after an animal was observed to have moulted. Crayfish showing excessively long intermoults were left out in the assignment to groups, but might be admitted later if the next intermoult were near the normal length. After intact controls had moulted a third time they were reassigned to whichever of the three experimental groups they happened to fall into. This resulted in the intact group being depleted of its more rapidly moulting members, which passed into and were recorded with, one of the two operational groups. As a result, the average intermoult for the remaining intact animals is abnormally long, and cannot be compared with the shorter pre-operational intermoults of the other two groups which will be discussed below.

No retinal removals were performed in the last 15 days of the experiment, while no eyestalks were removed in the last 11 days. There were thus more eyestalkless than retinaless animals recorded. A few eyestalkless animals were observed for 3 weeks after regular observations ceased in order to determine their survival times. The complete record of the intermoults observed in the two groups which had eyestalks and retinas removed is given in Tables I and II respectively. The intermoults which will be used to show the effects of eyestalk or retinal removal are the intermoults immediately before and following the operation.

In the group of crayfish whose eyestalks were removed, the intermoults immediately preceding operations averaged 12.13 $\pm$ 0.65 days, while the first intermoults following the operation averaged 8.10 $\pm$ 0.19 days (Table I). That this difference is significant is shown by the fact that the standard error of the difference of the means is 0.68 days, one-sixth the difference of the means. Of the 45 animals operated upon, only one died as a result of the operation, while a second has been disregarded in the calculations of mean intermoult and survival time because it is so obviously at variance with the rest. Twenty-two animals passed through a second intermoult after eyestalk removal. With two exceptions these second intermoults are as low as the first ones after the operations (Table I). Three animals moulted three times in the absence of their eyestalks. These third intermoults were

TABLE I

Complete record of intermoults observed in the group which had both eyestalks removed, and the survival times of those animals which died naturally following eyestalk removal.

Pre-operational Intermoults (length in days)			Post-operational Intermoults (length in days)			Survival Time (days)
Earlier pre-operational intermoults		Last pre-operational intermoult	First post-operational intermoult	Second post-operational intermoult	Third post-operational intermoult	
		15.5	7.5	7.0		[30–31]
		10.0	8.5	8.5		18.0
		9.5	8.5	8.5		[25–30]
		11.0	8.5			Fixed
	15.5	14.0	8.0			23.0
	11.5	9.0	6.5			Fixed
		11.5	7.0	7.0		Fixed
	10.5	9.0	7.5			Fixed
		11.0	6.5			Fixed
		14.5	7.0			12.5
	16.5	16.5	7.5	8.0	11.5	26.5
		14.5	7.0			Fixed
	10.5	11.0	8.5	9.5		Fixed
		12.5	7.5			Fixed
		12.0	7.5	8.0	10.5	26.0
	[13.0]	[9.0]	[16.0]	[12.0]		[45]
		10.0	7.0	7.0		Fixed
	13.0	11.0	7.0	8.5		[25–30]
		13.0	7.0	7.5	11.0	26.5
		14.0	11.0			19.5
14.0	9.0	10.0	8.5	9.0		[22–27]
		13.5	7.5			Fixed
	14.0	12.0	9.5			9.5
		16.0	7.5	9.0		[16–21]
	14.0	11.5	8.0	9.5		Fixed
		13.0	Died			——
		13.5	12.0			12.0
12.0	8.0	11.0	7.0	[22.0]		28.5
	11.0	[20.0]	8.0	9.0		26.5
		11.0	7.0	8.0		22.5
		10.0	7.5			13.0
		11.0	8.5			Fixed
		16.0	8.5			Fixed
	11.0	11.5	8.0			Fixed
	13.5	11.5	7.5	7.5		[20–25]
	12.0	14.5	7.5	7.5		[22–27]
	14.0	13.0	11.0			11.0
		13.5	8.5			Fixed
	11.0	10.0	7.5	8.0		22.0
		15.0	10.0			10.0
		13.0	8.0	10.0		28.0
	9.5	9.0	8.0			Fixed
	10.5	10.0	10.0			17.0
		10.5	10.0	9.0		18.0
13.0	11.94 ± 1.16	12.13 ± 0.65	8.10 ± 0.19	8.30 ± 0.17	11.0	19.47 ± 1.48

TABLE II

Complete record of intermoults observed in the group which had both retinas removed.

Pre-operational Intermoults (length in days)		Post-operational Intermoults (length in days)		
Earlier pre-operational intermoult	Last pre-operational intermoult	First post-operational intermoult	Second post-operational intermoult	Third post-operational intermoult
	16.5	Died		
12.0	9.0	9.5		
13.0	7.5	7.5		
	13.5	17.5		
15.5	12.5	14.0		
	10.5	17.5		
	15.5	Died		
10.5	8.0	12.5		
	13.0	9.5	9.5	
	14.5	20.0		
15.5	10.5	17.5		
	11.5	16.0		
	10.5	11.5	13.5	
10.0	12.5	10.0	11.5	
	9.5	12.0	13.0	
	13.0	12.5	12.0	15.5
11.0	17.0	12.5		
13.0	13.0	14.0		
	13.0	Died		
11.5	8.0	13.0	9.5	
9.5	11.0	14.0		
11.5	12.0	14.5		
	14.0	16.5		
	16.0	9.5		
	12.5	12.5	11.0	
13.0	10.0	14.0	14.0	
12.0	14.5	11.5		
14.5	8.5	15.5		
11.5	11.0	21.0		
7.5	10.5	18.5		
	13.5	14.5		
	9.5	Died		
	9.5	10.5	9.0	
11.0	10.5	21.0		
11.91± 0.50	11.82± 1.04	14.02± 0.62	11.44± 0.58	15.5

longer than the preceding ones, but as eyestalkless animals eat a great deal, and become distinctly larger than normal animals of the same age, this increase is not surprising. The number of second and third intermoults of eyestalkless animals would have been greater had not

about a third of the animals been fixed for histological examination at the end of the first intermoult after eyestalk removal.

The effects of retinal removal are in contrast to those of eyestalk removal. With a few exceptions the post-operational intermoults are longer than the preceding ones (Table II). Intermoults in 30 retinaless crayfish average 14.02 ± 0.62 days, while the intermoults just before the operations average 11.82 ± 1.04 days. The increase in the length of intermoult after retinal removal is, however, of doubtful significance, since the difference between the means is only 1.8 times the standard error of the difference. In a few cases a shortened intermoult followed retinal removal. It is difficult to remove completely the retinal zone of the eyestalk without including some ganglionic material, leading one to suspect that in these cases the sinusgland may have been removed as well as the retina. The severity of retinal removal is shown by the fact that 4 deaths resulted from 34 operations. However, the remaining animals continued perfectly healthy, no other deaths occurring during the whole observation period. Retinaless animals did not attain the size of eyestalkless animals, a fact probably related to their slower rate of moulting. They tended to become quite dark in color, in contrast to the eyestalkless animals, which took on a pronounced reddish-brown color, becoming much paler after ecdysis.

Discussion

The results obtained show that eyestalk removal causes a shortening of the following intermoults in young crayfish. The mechanism suggested by Brown and Cunningham (1939) and Hanström (1939), that the eyestalks normally produce a hormone tending to delay or inhibit moulting, seems to be a reasonable explanation.

It has been shown that even severe injury does not shorten the intermoult, at least when the injury occurs early in the intermoult. Darby (1938) has stated that operative injury appears to hasten the next moult in *Crangon armillatus*. Possibly, however, his results may mean that the shock of injury late in the intermoult period causes a speeding up of moulting processes already well advanced in the animal, while injury early in the intermoult may have no such effect. It may be necessary to distinguish between the acceleration of moulting caused by eyestalk removal and that reported by Darby as caused by other types of injury.

The observation of Abramowitz and Abramowitz (1940) that *Uca* may occasionally moult more than once after eyestalk removal has been found to be true also for *Cambarus*, where three intermoults have been recorded after eyestalk removal. It seems beyond question that

the processes leading up to moulting can be initiated in the absence of the eyestalks, while "shock" effects are ruled out as a cause of the shortened intermoult.

A comparison of the survival time of eyestalkless animals in the second experiment with that of those in the first reveals a curious discrepancy. In 1938, with cold water (9°–14° C.) and rather scanty food, the average intermoult among eyestalkless animals was 15.9 days, the average survival 17.1, or at most 19.7 days. In 1939, with warm water (20°–22° C.) and unlimited food, the average intermoult in eyestalkless animals fell to 8.1 days, as might be expected, but survival averaged 19.5 days. Although not proved, it seems probable that the abundant food supply increased the expected survival time in 1939 over that in 1938. Otherwise survival in 1939 would have been shortened by warmth and favorable conditions to the same extent that intermoults were shortened. This observation, that in eyestalkless animals shortening of intermoult and time of survival are not necessarily parallel effects, suggests that the processes related to viability in the eyestalkless crayfish may be separated from those processes which underlie the more rapid moulting of such animals. However, no evidence so far presented is sufficient to show whether these processes are controlled by one substance from the eyestalks, or by two or more.

The fact that eyestalkless crayfish may moult successfully shows that the aid of the eyestalks is not indispensable to the physiological changes occurring at ecdysis, but that the moulting processes may be seriously interfered with by lack of the eyestalks is suggested by Abramowitz and Abramowitz (1940), who report that most of the deaths after eyestalk removal follow close upon a moult. This can be verified from the writer's second experiment. Twenty animals were maintained after eyestalk removal until they died. Table III shows that deaths fall into two main groups. The first is directly after the time of moulting, the second about 8 days later. Since the average

TABLE III

Survival time of eyestalkless crayfish after the last moult

Survival time in days	0*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Number of deaths	2	6	2	—	—	—	2	—	2	2	1	—	1	—	—	—	1	1

* Died in moult.

intermoult of eyestalkless animals is a little over 8 days, it seems probable that the second group of deaths is that of animals which died in an incipient moult, and suggests that the physiological changes associated

with moulting impose a severe strain which the animal fails to meet without the aid of some factor from its eyestalks. Presumably the weaker animals die before accomplishing ecdysis, while stronger ones may live long enough to moult, and still others may "recover" and pass through the next intermoult, to fail at some future moult. A few eyestalkless animals lived longer than 10 days after their final moults. This may possibly be because they were in such a weakened state that the next (incipient) moults were considerably delayed.

Summary

1. Removal of both eyestalks causes a shortening of the following intermoults in *Cambarus clarkii*. The removal of a moult-inhibiting substance produced in the eyestalks is accepted as a reasonable explanation.

2. The methods employed have been such as to show: (*a*) that the processes leading up to moulting can be initiated in the absence of the eyestalks; and (*b*) that the effect upon moulting is associated with the absence of the eyestalks, not with the shock of their removal.

3. Injury other than eyestalk removal does not shorten the intermoult when performed early in the intermoult.

4. Eyestalk removal always results in death, but the processes related to viability which are affected by eyestalk removal can be distinguished from the moulting processes which are affected by the same operation, although there is an apparent correlation between moulting and viability.

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EVIDENCE FOR THE PROTEIN NATURE OF THE SPERM AGGLUTININS OF THE KEYHOLE LIMPET AND THE SEA-URCHIN

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Chemical investigations on agglutinins (both naturally occurring and immune) for blood cells and bacteria have furnished strong evidence for the view that they are always of protein nature (see Marrack, 1938). It might be expected, then, that the sperm agglutinins present in the egg water of certain species of marine animals (see Lillie, 1919; Lillie and Just, 1924; Just, 1930; Tyler, 1940) would also show protein properties. This was not found to be the case by the earlier investigators. Glaser (1914) and Woodward (1918) applied a number of common protein tests to sea-urchin (*Arbacia*) egg water and obtained no reaction with the exception of a weak, partial xanthoproteic test. This does not, however, eliminate the possibility that the agglutinin is of protein nature, since it has often been shown that physiological responses can be evoked by solutions of proteins too dilute to give the ordinary tests. In fact, proteins can be detected in serological reactions at dilutions at which the color tests fail. For example, Uhlenhuth (1909) showed that a dilution of a protein as high as 1 to 100,000 will give a detectable reaction with anti-serum whereas neither the Biuret nor the Millon's reaction will exceed 1 to 10,000. Agglutination reactions are many times more sensitive than precipitin reactions (Zinsser, 1939; p. 246) and anaphylactic reactions still more sensitive.

In a preliminary note (Tyler and Fox, 1939) evidence that the sperm agglutinins of the keyhole limpet and of the sea-urchin are proteins has been reported. The present article presents the details of this evidence and further information on the properties and possible methods of purification of the agglutinins.

Bioassay

The agglutination reaction in the keyhole limpet, *Megathura crenulata*, has recently been described (Tyler, 1940*a*) in some detail. It was shown that the time at which agglutination of the sperm first becomes macroscopically visible increases with increasing dilution of the egg water (agglutinin), within certain limits and for a given sperm

suspension. This relation between concentration and agglutination time can be employed in a bioassay. For this purpose an arbitrary agglutination time is taken as a standard and the dilution of the test solution that gives the standard agglutination time is determined. The reciprocal of the dilution factor gives the agglutinin titer in arbitrary units. Since with concentrated solutions the agglutination time changes very much less than with dilute solutions, it is advisable to take as a standard a reaction obtained in a relatively dilute solution. We have usually taken as a standard a reaction that is first visible macroscopically in 30 seconds when equal volumes of the agglutinin solution and a 1 per cent sperm suspension are mixed. Solutions giving the standard reaction time are designated as having one unit concentration of agglutinin. The unit concentration selected is well above the weakest solution that gives a perceptible reaction. The latter end point, however, is not as easily determined. Sperm suspensions from different animals as well as suspensions of different ages and history from the same animal will, of course, show considerable variation in their reaction time even when employed in closely similar concentrations. The units have only approximate significance when comparisons of different series of tests are made. In most instances, however, the comparisons are made between control and test solutions simultaneously on samples of the same sperm suspensions. The concentration of agglutinin in the test solution is then given as a fraction or percentage of that in the control.

Other methods of bioassay that were tried with the keyhole limpet included determining the number and size of the clumps of agglutinated sperm and centrifuging in a hematocrit tube. These two methods did not prove as convenient nor as reproducible as the method of determining the time for agglutination to become macroscopically visible.

The agglutination reaction in the sea-urchin, *Strongylocentrotus purpuratus* (Loeb, 1914; Lillie, 1921), is quite similar to that in *Arbacia* (Lillie, 1913). The reaction occurs so rapidly even with dilute agglutinin solutions that it is not feasible to use the time at which it first becomes visible for bioassay. The method employed by Lillie (1914) is preferable. He showed that in the sea-urchin the agglutination reverses (i.e. the sperm disperse) within a short period of time ranging from a few seconds to a few minutes. The time at which reversal occurs decreases with decreasing concentration of the egg water. Lillie defined as a unit a solution of such concentration that the reaction reverses within three to five seconds, the observations being made under the microscope. For greater convenience we employed, as a standard, a reaction that reverses in one minute, the observations

being made macroscopically. A solution which, when mixed with an equal volume of a 1 per cent sperm suspension, gives a reaction that lasts one minute, is then designated as having one unit concentration of agglutinin.

Extraction

As Lillie first showed with *Arbacia* and *Nereis*, it is unnecessary to treat the eggs in any special manner in order to obtain agglutinin solutions. The sea water above the eggs becomes charged with the substance, the concentration increasing continuously with time. Allowing eggs to age in sea water is, however, not a very satisfactory method for obtaining solutions of high agglutinin titer. The eggs slowly disintegrate as they remain in sea water. This not only encourages bacterial growth and adds to the impurities but also liberates an anti-agglutinin (Lillie, 1914; Tyler, 1940*b*).

The method we have employed consists simply in dissolving the jelly layer surrounding the egg by means of acidified sea water. As was shown in the previous article, the agglutinin is either the jelly substance itself, or a component of it. The jelly slowly dissolves as the eggs age in ordinary sea water. In the sea-urchin, sea water acidified to pH 3.5 almost immediately dissolves the jelly layer without injuring the rest of the egg. A single extraction gives a solution containing practically all of the agglutinin that can be obtained from the eggs. A suspension of about 10^8 eggs in 100 cc. of acid sea water gives a solution of 32 units. Different preparations, however, vary widely in the yield.

In the keyhole limpet, the jelly layer does not dissolve as rapidly in acidified sea water as in the sea-urchin. There is, however, a rapid swelling and softening of the jelly and the eggs can be readily centrifuged out of their jelly hulls in the acid sea water. By this means agglutinin solutions of very high titer are rapidly obtained in the keyhole limpet too.

The agglutinins can also be rapidly extracted with isotonic NaCl acidified to pH 3.5. This was employed in some experiments in which it was desirable to eliminate certain of the sea water salts.

Rather weak or inactive preparations are obtained by such methods as freezing and thawing or extraction with distilled water which entail cytolysis of the eggs. This is due to the inactivation of the agglutinin by an anti-agglutinin (recently isolated by Tyler, 1940*b*) present within the eggs.

Extraction of the eggs by alcohol, acetone or ether failed to give active preparations. Also the agglutinin was not extractable from active precipitates (see below) or solutions by means of these solvents.

Dialysis

Lillie (1914) showed that the sperm agglutinin of *Arbacia* is non-dialyzable. We have found the same to be true for the sperm agglutinins of *Strongylocentrotus* and of *Megathura*. Concentrated solutions (approximately 20 to 30 units) of the agglutinins were placed in cellophane tubes or in collodion bags and dialyzed against approximately two to three times the volume of ordinary sea water with constant stirring. The dialysis was carried out in a cold room at 1° C. Samples were tested at regular intervals up to two weeks. The dialysate in all cases was completely inactive while the solutions within the tubes or bags retained practically their original activity during this period. Other samples that were dialyzed against running sea water likewise showed no loss in activity. It is evident, then, that the sperm agglutinins of the sea-urchin and of the keyhole limpet are substances of large molecular size.

Precipitation

It has been reported by Woodward (1918) that the sperm agglutinin of *Arbacia* partially precipitates when the egg water is saturated with $(NH_4)_2SO_4$. In *Strongylocentrotus* and *Megathura* we find that the agglutinins can be practically completely precipitated by the addition of $(NH_4)_2SO_4$.

When concentrated egg water of *Strongylocentrotus* or of *Megathura* is saturated with $(NH_4)_2SO_4$ a white flocculent precipitate slowly appears. The precipitation is usually complete within about 24 hours. The initial egg waters are usually opalescent in appearance. After removal of the precipitates the supernatants appear quite clear. Precipitates obtained from *Strongylocentrotus* and from *Megathura* egg waters were dissolved in sea water, dialyzed against running sea water to remove the $(NH_4)_2SO_4$, and adjusted to the original volumes. Agglutination tests with these solutions showed in all cases no significant differences from the original solutions. The supernatant solutions were also dialyzed and tested. With none of the latter was any sign of agglutination obtained. The following table gives the results of three such experiments with the keyhole limpet and two with the sea-urchin. The figures represent units concentration of agglutinin as defined above (p. 154).

	Megathura			*Strongylocentrotus*	
	A	*B*	*C*	*A*	*B*
Original egg water	16	8	6	32	24
Precipitate	12	10	8	32	20
Supernatant	0	0	0	0	0

It is evident that, in both *Megathura* and *Strongylocentrotus*, the agglutinin is completely salted out by saturation with ammonium sulfate.

An approximate determination was made, with *Megathura* egg water, of the lowest concentration of ammonium sulfate required for complete salting out of the agglutinin. To four 100-ml. portions of an approximately 10 unit agglutinin solution, made up in isotonic (0.55M) NaCl at pH 3, were added 30, 40, 50 and 55 grams of ammonium sulfate respectively. The salt dissolved completely in the first three and a small amount remained undissolved in the fourth. After standing 24 hours at room temperature the second, third and fourth flasks showed flocculent precipitates and clear supernatant solutions. The first flask showed no precipitate and the solution remained opalescent. The agglutinin titers of the precipitate and the supernatant in the second flask (40 grams) were determined after dialysis and adjustment to original volume. The precipitate gave practically the same agglutinin titer as the original solution; the supernatant showed no activity. Precipitation of the agglutinin is, then, complete in approximately three-fourths saturated ammonium sulfate.

A preliminary attempt at fractionation was made by slowly increasing the concentration of ammonium sulfate and removing the precipitate that first appears (at about 2/3 saturation) separately from that which comes out at higher saturation. Both fractions showed activity and, based on the relative centrifuge volumes of the precipitates, no marked difference in agglutinin titer was manifest.

Preliminary attempts at crystallization were also made with material prepared by dialysis, adsorption (see below), and repeated precipitation with 2/3 to 3/4 saturated ammonium sulfate by allowing the precipitation to take place in a dialyzing bag immersed in a solution of ammonium sulfate, the concentration of which was very slowly increased; also by slow evaporation of ammonium sulfate solutions of the agglutinin. In none of these were recognizable crystals obtained.

Adsorption

The agglutinins of both *Megathura* and *Strongylocentrotus* can be completely removed from solution by solid $CaCO_3$. For elution the $CaCO_3$ is dissolved by the addition of acid. This involves the disadvantage that some or all of the agglutinin may be inactivated by the acid (see below). To avoid this, it is necessary to employ a concentration of acid just sufficient to dissolve the $CaCO_3$ at a reasonable rate but not strong enough to inactivate the agglutinin. By use of sea water acidified to about pH 2.5 to 2.8 we have recovered approximately 25 to 50 per cent of the original agglutinin. Other methods of elution have not as yet been investigated.

The agglutinins are also adsorbed by Al_2O_3, charcoal and kaolin.

Color Tests

Several of the common color tests for proteins were applied to concentrates of sea-urchin and keyhole limpet agglutinins. The material was prepared by dialysis and ammonium sulfate precipitation of egg water obtained with the utmost care to avoid injury to the eggs themselves. In the case of the sea-urchin, it is quite feasible to prepare a concentrated egg water without any injury to the eggs and also to avoid or get rid of body fluid contamination. In the keyhole limpet this is more difficult unless one employs naturally shed eggs (see previous article). Definitely positive xanthoproteic, biuret and Millon's reactions were obtained with the agglutinin preparations from both sea-urchin and keyhole limpet. For the Millon's test it is necessary to dialyze against distilled water (washing the $(NH_4)_2SO_4$ precipitate on a membrane filter suffices) in order to remove salts, particularly chlorides, that interfere with the reaction. While the distilled water generally inactivates the agglutinin, this does not by any means invalidate the test as an indicator of the presence of protein. Attempts were also made to obtain sugar tests on portions of the material heated in concentrated HCl. These were all negative.

The failure of the earlier workers (Glaser, 1914; Woodward, 1918) to obtain positive protein tests with *Arbacia* egg water may very likely be due to their having worked with much weaker preparations. Positive reactions to these color tests do not, of course, prove that the agglutinin is of protein nature. They do, however, show the presence of proteins in the preparations. Since the method of obtaining the egg waters involves practically no destruction of the eggs themselves but simply dissolves the jelly layer, it is unlikely that there are very many different substances of high molecular weight present in the preparations. Previous evidence (Tyler, 1940*a*) had shown that the agglutinin is either the jelly substance itself or if the jelly is composed of more than one substance, that it is a component of it. If it were known that only a single high molecular weight substance were present in the preparations, then the color tests would be convincing evidence of the protein nature of the agglutinin. In place of such information other kinds of evidence have been obtained.

Action of Enzymes

Evidence for the protein or polypeptide nature of a substance may be obtained by digestion with proteolytic enzymes. The evidence is not very satisfactory when crude preparations of the proteinases are

employed since in such preparations there are generally other enzymes present. In recent years, however, a number of proteolytic enzymes have been crystallized (see Northrop, 1939), and these are, of course, eminently suitable for the tests. We have obtained, through the courtesy of Dr. J. H. Northrop, samples of crystalline trypsin and chymotrypsin and have examined their action on the sperm agglutinins of the keyhole limpet and the sea-urchin.

The *Strongylocentrotus* agglutinin is inactivated fairly rapidly by both trypsin and chymotrypsin. In four experiments that were run, inactivation was practically complete in less than 3 hours. The following figures give the agglutinin titers in one of the experiments in which solutions of trypsin, chymotrypsin and also steapsin (commercial) all adjusted to pH 8.0 were added to equal volumes of egg water, allowed to act at 20° C. and samples tested at the times indicated.

	2 min.	1 hour	2 hours	3 hours
Control	8	8	8	8
Saturated trypsin	8	6	3	0
1 per cent chymotrypsin	8	1/2	0	0
Saturated steapsin	8	8	6	6

The slight inactivation obtained with the commercial steapsin is perhaps to be attributed to small amounts of other enzymes present as impurities. The inactivation of the agglutinin by trypsin and by chymotrypsin occurs more rapidly than might be expected on the basis of the rate at which proteins are in general hydrolyzed by these enzymes. However, it is not necessary to assume that the substance must be split by the enzymes in order for inactivation to occur. In enzyme reactions there is generally considered to be an intermediate addition compound first formed. If, in the case of the agglutinin, the initial combination involved those groups that are concerned with its reaction with the sperm, there would be, in the presence of sufficient enzyme, a very rapid inactivation.

The *Megathura* agglutinin is much more slowly inactivated by solutions of the crystalline proteinases. In three experiments that were run, about seven days were required to reduce the titer to a point where no definite agglutination reaction is obtained. It is important, therefore, to have reasonably sterile conditions. This is relatively easy to do in the case of the keyhole limpet agglutinin since the solutions can withstand boiling for a considerable time (see below). The following figures (agglutinin concentration units—see bioassay section) give the course of the inactivation in one experiment run at pH 8 and 20° C.

	5 min.	1 day	2 days	3 days	5 days	7 days	8 days
Control	16	14	16	16	14	12	12
Saturated trypsin	16	14	12	12	10	1/2	0
1 per cent chymotrypsin	16	16	10	6	1/2	0	0

In this, as well as the other two experiments, the inactivation sets in slowly during the first few days and then proceeds more rapidly thereafter. Assuming that here, too, the initial combination of enzyme with substrate is quite rapid, it appears that this does not involve those groups on the agglutinin molecule which enable it to react with the sperm. The inactivation must then occur during the actual digestion of the agglutinin.

It would be desirable to determine whether or not there is a difference between the sea-urchin and the keyhole limpet agglutinins in regard to their manner of inactivation by these proteolytic enzymes. This could be tested by examining the digests for products of hydrolysis during the course of the inactivation. Due to limitations of material and to the desirability of using preparations that are of known purity for such purposes, the determinations have not as yet been made. One set of formol titrations has, however, been run on the digests (and controls) of the keyhole limpet agglutinin in the experiment listed above. The results showed that appreciable hydrolysis had occurred in the enzyme solutions. The determinations were made on aliquot samples removed on the tenth day. Solutions of trypsin and of chymotrypsin that had been kept under the same conditions as the digestion mixtures were added to samples of the control agglutinin solution at that time and the digestion mixtures were diluted correspondingly with sea water. Allowing for the controls the formol titrations gave 8×10^{-6} equivalents of $-COOH$ per ml. for the trypsin digest and 13×10^{-6} equivalents for the chymotrypsin digest.

Heat and pH Inactivation

The stability of solutions of both the keyhole limpet and the sea-urchin agglutinins varies with the temperature and the pH at which they are kept. Since the time for inactivation at a given temperature is a function of the pH of the solution, both of these variables may be considered together.

The sea-urchin agglutinin is fairly rapidly inactivated by heating the solutions. The following figures give the time required for half-inactivation and for practically complete inactivation of solutions of various pH at 100° C.

pH	2.50	3.1	4.2	6.3	7.3	8.2
Minutes for 50 per cent inactivation	.....	2–3	12–15	15–20	6–8	4–5
Minutes for 95–100 per cent inactivation	3	5–7	20–25	70–85	20–30	15–20

As the figures show, the agglutinin is most stable in the range of pH 4 to 7. At lower temperatures the time for inactivation increases, but the effect of pH remains substantially the same. Thus at 20° C. solutions at pH 4 retain their full activity for more than three days while pH 2 and pH 8 solutions are completely inactivated. At 0° C. solutions at pH 4 have been kept for over 6 months with no appreciable loss in activity.

When sea water solutions of the agglutinin are made alkaline (pH 9 and above), a precipitate of calcium and magnesium carbonates and hydroxides forms and this adsorbs the agglutinin. The agglutinin can be completely recovered by dissolving the precipitate in acid sea water. Due to the formation of the precipitate and adsorption of the agglutinin, sea water solutions could not be used for determining the rate of inactivation in the more alkaline range. Solutions in isotonic NaCl were employed for this purpose and these showed a continuous decrease in stability as the pH was raised.

The keyhole limpet agglutinin is considerably more stable in solution than is that of the sea-urchin. The following figures give the time required for half-inactivation and for nearly complete inactivation of solutions of various pH at 100° C.

pH	1.2	2.0	3.2	5.0	8.2	9.2	11.0
Hours for 50 per cent inactivation	1/6	1–1½	24–36	21–30	5–9	4–6	1/30
Hours for 95–100 per cent inactivation	1/2	3–3½	60–90	44–90	14–18	14–18	1/6

The determinations at pH 9.2 and 11.0 were made on solutions of the agglutinin in isotonic NaCl, since, as in the case of the sea-urchin, the precipitate that forms upon the addition of alkali to sea water adsorbs the agglutinin. Here again the agglutinin can be completely recovered by dissolving the precipitate. The pH range of maximum stability of the keyhole limpet agglutinin is roughly the same as that of the sea-urchin. It is, however, considerably more stable, being able to withstand boiling for more than 24 hours with only a 50 per cent loss in activity. At lower temperatures the stability varies in the

same manner with pH. Solutions of pH 2.5 to 5.5 kept at room temperature remain fully active for more than a week while solutions at pH 1 and pH 11 are completely inactivated in less than a day. At 0° C. the solutions of pH 2.5 to 5.5 have been kept more than 6 months with no loss of activity.

The inactivation of agglutinin solutions by heat may be considered to be due to a denaturation of the active substance. With concentrated solutions of both the keyhole limpet and the sea-urchin agglutinins a precipitate forms upon inactivation by heating. It is possible, however, to inactivate completely the agglutinins without the appearance of a precipitate or coagulum. This occurs generally with dilute solutions. The failure of a coagulum to appear upon inactivation of dilute solutions does not, however, exclude the possibility that the effect is due to denaturation of the substance. Denaturation is generally assumed to involve more than one step (see Mirsky, 1938), the final one being coagulation.

Dry Weights and Nitrogen Content

The concentration of organic matter was determined in agglutinin solutions of keyhole limpet and of sea-urchin prepared with special care to avoid injury to the eggs and contamination with body fluids, etc. Samples of the solutions were dialyzed against distilled water of known solid content, evaporated to small volume by boiling and dried at 80° C. to constant weight. Micro-Kjeldahl nitrogen determinations were then made on the dried material. The results were as follows:

		Megathura	*Strongylocentrotus*
Titer of Solutions		32 units	16 units
Organic solid content	range	0.10–0.14%	0.028–0.031%
	average	0.11%	0.03%
Nitrogen content	range	2.8–5.2%	3.8–5.9%
	average	4.6%	5.2%

The low content of organic solid matter may perhaps account for the failure of the earlier workers to obtain positive protein tests on sea-urchin agglutinin. The nitrogen content (about 5 per cent) is evidently too low for a pure protein. However, we do not as yet know how much inactive material may be present. The present values may be taken then merely as showing the presence of a definitely detectable quantity of nitrogen which is consistent with the other evidence that the agglutinin is of protein nature.

The amount of solid matter contributed per egg may be estimated roughly for the sea-urchin from the fact that a 16 unit solution is ob-

tained by extracting 5×10^7 eggs in 100 cc. of acid sea water. Since 100 cc. of the solution yields 30 mg. of organic solid matter, a single egg contributed 6×10^{-7} mg. In the absence of information as to the amount of inactive material present, this value may be taken as representing a maximum for the quantity of agglutinin obtainable per egg. The volume of an egg of *S. purpuratus* is 2.6×10^{-7} cc. and, assuming a density of 1.04, the wet weight would be 2.7×10^{-4} mg. From the figures of Leitch (1934), the dry weight would be about one-fifth of the wet weight or 5×10^{-5} mg., and roughly three-fourths of this is protein. The quantity of agglutinin obtained from a single egg would then correspond at most to 1 per cent of the dry weight of the egg.

Discussion

From the method of extraction, the non-dialyzability, precipitation with $(NH_4)_2SO_4$, the color tests, the heat- and pH-lability, the insolubility in alcohol and ether, the presence of nitrogen and particularly the inactivation by means of purified proteinases, one may conclude that the sperm-agglutinin both of the sea-urchin and of the keyhole limpet is either protein or closely associated therewith. This is consistent with the results on other naturally occurring agglutinins as well as immune agglutinins (and antibodies in general) that have been investigated (see Marrack, 1938; Heidelberger, 1938; Landsteiner, 1936; Zinsser, 1939). It is also in line with the specificity of the reaction.

The other active substances that have been isolated from sperm and eggs: namely, the anti-agglutinins, egg agglutinins and egg membrane lysin (Tyler, 1939, 1940*b*), also appear to be of protein nature. It would seem, then, that there is some justification for the analogies which Lillie drew between the fertilization reaction and immunological reactions. However, considerably more work will be necessary in both fields before we can determine to what extent immunological principles may be used to interpret the fertilization reactions.

The sperm agglutinins of the two animals investigated differ markedly, as the results show, in certain properties. That of the keyhole limpet is considerably more resistant to inactivation by heat (and pH change) and by proteolytic enzymes than is that of the sea-urchin. The situation is, however, not unusual. Marrack (1938, p. 50) reports that the heat stability of agglutinins (and antibodies in general) is very variable. Even in the same sera considerable differences in lability between different agglutinins have been found. Different antibodies also differ in regard to their resistance to the action of proteolytic enzymes (Marrack, p. 52). The destruction proceeds in

many cases quite slowly and in some instances use has been made of the relative resistance to digestion in attempts to purify antibodies (see Zinsser, p. 172).

The difference in stability between keyhole limpet and sea-urchin agglutinins correlates with differences in the nature of the reaction in these two forms. It has been previously shown (Tyler, 1940*a*) that the sperm agglutination reaction in the keyhole limpet persists considerably longer than does the reaction in the sea-urchin. Corresponding to the difference in duration of the reaction, a difference was found in the time at which precipitation occurs when the active principle (anti-agglutinin) from sperm is added to agglutinin solutions. In both species inactivation of the agglutinin occurs immediately after addition of the anti-agglutinin but while precipitation occurs within a few minutes in the sea-urchin it is many hours later in the keyhole limpet. These differences are not necessarily attributable entirely to the agglutinins, since the properties of the sperm and the sperm extracts cannot be assumed to be the same in the two species. Nevertheless, it is of interest to note that if greater stability is assumed to mean also slower reactivity on the part of the substance, then the differences in the properties of the keyhole limpet and sea-urchin agglutinins reported here afford a reasonable interpretation of the difference in the agglutination reaction in these two forms. In other words, the reactivity of the keyhole limpet substance may be considered to be lower than that of the sea-urchin.

Summary

Some of the chemical and physical properties of the sperm agglutinin (fertilizin) of the keyhole limpet *Megathura crenulata* and of the sea-urchin *Strongylocentrotus purpuratus* were investigated. In both species the agglutinins were found to be non-dialyzable. They precipitate completely without loss of activity in nearly saturated ammonium sulfate. They are adsorbed by $CaCO_3$, Al_2O_3, charcoal and kaolin. The agglutinins are insoluble in alcohol and ether. Active concentrates give, contrary to the findings of earlier investigators, the common color tests for proteins and are found to contain nitrogen. Solutions of crystallized proteinases (trypsin and chymotrypsin) inactivate the agglutinins. They are also inactivated by heat as a function of pH. From the evidence it is concluded that in both species the agglutinating principle is either protein or very closely associated with protein.

The keyhole limpet agglutinin is much more resistant than is that of the sea-urchin to inactivation by heat and by proteolytic enzymes.

It is suggested that the difference between the two species in the duration of the agglutination reaction may be related to the difference in stability of the respective agglutinins.

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A COMPARISON OF THE DEVELOPMENT OF NUCLEATE AND NON-NUCLEATE EGGS OF ARBACIA PUNCTULATA

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A further study has been made of the development of the non-nucleate half-eggs or parthenogenetic merogones of *Arbacia punctulata*, especially in comparison with similar nucleate half-eggs or fertilized merogones. A study has also been made of the cytological details as shown in sections of fixed material, and of the reaction with the Feulgen technique. In order to have a better understanding of the development of the parthenogenetic and fertilized merogones (red halves), a further study has been made of the fertilized nucleate half-eggs (white halves) in comparison with similar whole eggs both normal (uncentrifuged) and centrifuged. Differences in the development of these eggs and half-eggs are caused not only by differences in nuclear content, whether both ♂ and ♀ nuclei are present, or only one, or none at all, but also by the shape of the eggs and differences in cytoplasmic content caused by the centrifugal force.

This comparative study is presented in a series of photographs arranged especially for comparison, and the reader is requested to study the plates which are almost self-explanatory.

Material and Methods (Plate I)

The normal *Arbacia* egg when centrifuged (3 minutes at 10,000 × g) stratifies, elongates, becomes dumb-bell shape and then breaks into halves; these halves with further centrifuging elongate and break into quarters. This is shown in Plate I (Photographs 1–11), and has been described in previous papers (1932, 1936). The important facts are that the nucleus always goes intact to the light pole under the oil

EXPLANATION OF PLATES

The photographs are all of living eggs, except those in Plate VIII, which are of sections. The photographs of Plates I to VI (except 64) are all as nearly as possible of the same magnification, approximately 250×. The photographs of Plate VII and 64 are also all to the same magnification, approximately 60×. The photographs of Plate VIII were taken with an oil immersion lens and magnified approximately 400× as presented, except 130 and 131, which are magnified about twice that amount. The times given under each photograph refer to the time after fertilization or activation at 23° C.

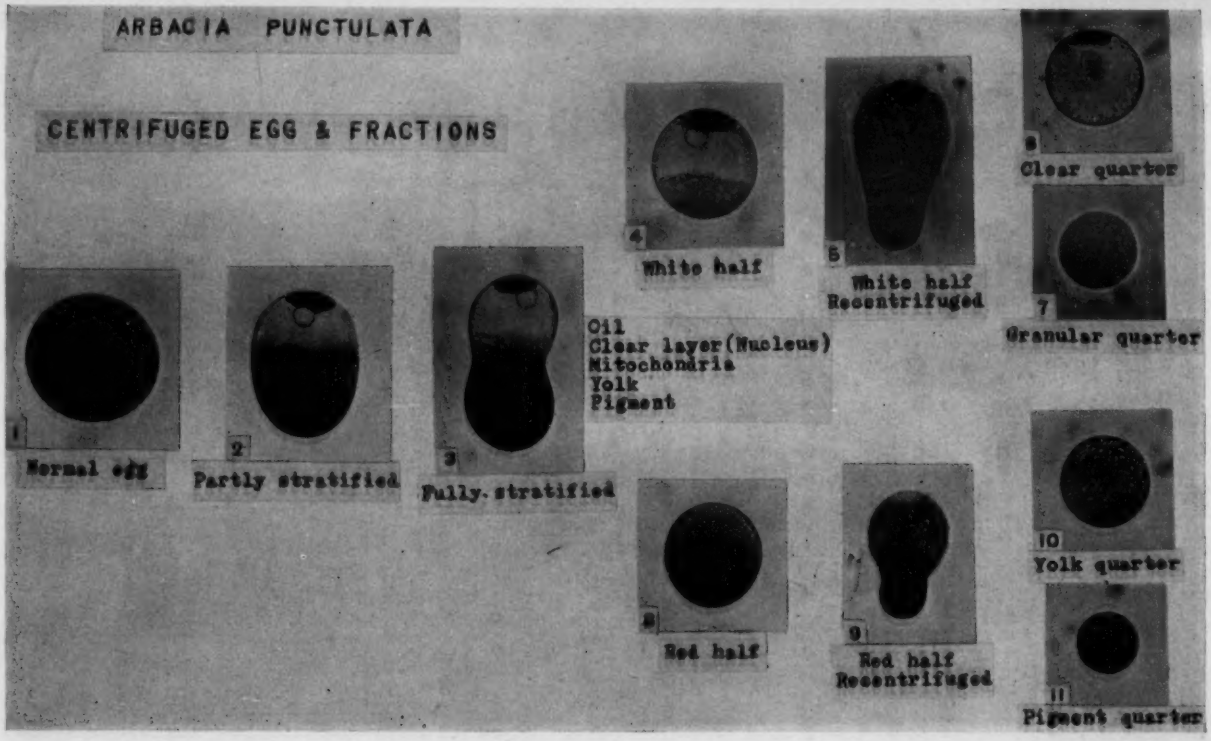

PLATE I

Centrifuged egg and fractions

The actual sizes (diameters) are as follows: whole egg 74 μ, white half 62 μ, clear quarter 56 μ, granular quarter 40 μ, red half 56 μ, yolk quarter 52 μ, pigment quarter 32 μ. (See 1932 paper.)

cap, and that the halves and quarters are uniform in size, but differ from each other in cytoplasmic content as well as in size. All of the halves and quarters can be fertilized; all will throw off a fertilization membrane and at least begin development. When the elongate whole egg and the elongate halves are fertilized, they become "set" and retain their shape. If left for an hour or so in sea water, unfertilized, they become spherical and the granules partially redistribute. The white and red halves develop much better if allowed to stand an hour or so before fertilizing them.

The whole eggs, both normal (uncentrifuged) and centrifuged, will develop parthenogenetically if treated for 20 minutes with a hypertonic salt solution made by boiling sea water to half its volume or by adding 30 grams of NaCl per liter of sea water. The white (nucleate) halves develop parthenogenetically with practically the same treatment, and the red (non-nucleate) halves also develop to a certain stage. It is of interest that a *hypo*tonic solution will cause parthenogenesis as well as a *hyper*tonic solution; the immersion of the sea-urchin egg for about a minute in distilled water will cause activation (Schücking, 1903); but the eggs of *Arbacia punctulata* develop only to the amphiaster of the first cleavage and only rarely cleave with this treatment.

Study of Development

Whole Egg, Centrifuged then Fertilized (*Plate II, 12–15*)

The elongate centrifuged whole egg, when fertilized immediately, develops as shown in Photographs 12–15. The chief points of interest are that the cytoplasmic materials remain partially segregated and the first cleavage plane comes across the short axis in a rather definite position, so that the first two cells are unequal. The pigmented cells are throughout the cleavages, generally larger than the unpigmented. Micromeres have not been observed. In spite of the peculiar cleavages,

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Plate II

Centrifuged and normal egg

Photographs 12–15. Centrifuged egg, fertilized immediately.
Photographs 16–32. Normal (uncentrifuged) egg.
Photograph 17. Monaster stage.
Photograph 18. "Streak" stage.
Photograph 19. Nuclear membrane just broken.
Photograph 20. Amphiaster.
Photograph 22. Immediately after first cleavage; cells well separated.
Photograph 23. Just before second cleavage; cells close together.
Photograph 26. Micromere stage; 12 cells.
Photograph 28. Blurring caused by swimming of blastula inside membrane.
Photograph 29. Hatching from fertilization membrane.
Photograph 30. Late blastula; note cilia. The animal was narcotized.
Photograph 31. Gastrulation has begun. Note cilia.
Photograph 32. Well-formed pluteus. Notice lattice-like skeleton in arms.

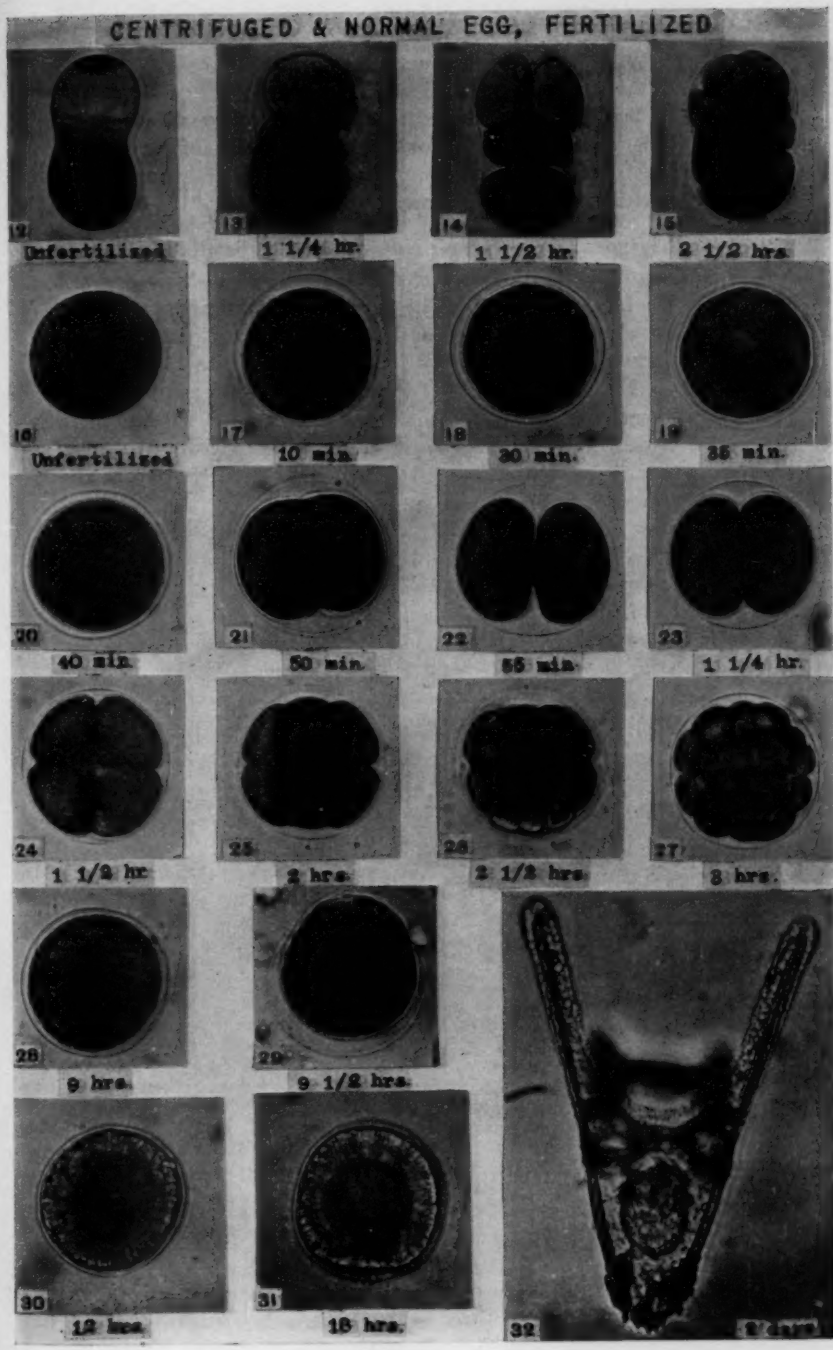
CENTRIFUGED & NORMAL EGG, FERTILIZED
12
Unfertilized
13
1 1/4 hr.
14
1 1/2 hr.
15
2 1/2 hrs.
16
Unfertilized
17
10 min.
18
30 min.
19
35 min.
20
40 min.
21
50 min.
22
55 min.
23
1 1/4 hr.
24
1 1/2 hr.
25
2 hrs.
26
2 1/2 hrs.
27
3 hrs.
28
9 hrs.
29
9 1/2 hrs.
30
12 hrs.
31
18 hrs.
32

PLATE II

which result in slipper-shaped blastulae, plutei are formed which are normal in every respect except for the concentration of pigment in certain areas. Usually the pigment is near the oral end, but it may be in any position, as originally described by Lyon (1907).

Normal (Uncentrifuged) Whole Egg, Fertilized (Plate II, 16–32)

Some stages in the development of the normal *Arbacia* egg are shown in Photographs 16–32. The especial characteristics are that the first three cleavages are equal and that at the next cleavage micromeres are formed, small colorless cells, giving a definite 12-cell stage (Photograph 26). A peculiarity following the first cleavage is that the two cells are at first widely separated (Photograph 22), and later become pressed together, probably owing to the formation of the next mitotic figure (Photograph 23). The blastulae hatch out from the fertilization membrane when having some 500–600 cells (computed from photographs),[1] and they have only a small blastocoel. The skeleton of the *Arbacia* (*punctulata* and *pustulosa*) pluteus is the lattice type like that of *Sphaerechinus granularis* and unlike the simple rods of *Paracentrotus lividus*, *Parechinus microtuberculatus* and *Strongylocentrotus dröbachiensis*.[2]

Whole Egg, Parthenogenetic

Both the normal (uncentrifuged) and the elongate (centrifuged) egg will develop parthenogenetically similarly to the fertilized egg but more slowly. Plutei have been obtained in both cases.

[1] This agrees fairly well with Morgan's (1895 *a*, *b*) estimate of 500–526 cells for *Sphaerechinus granularis* and 1,000 for *Echinus microtuberculatus*, and MacBride's (1914) figure of 808 for *Echinus esculentus*, at the time of hatching. The number of cells computed for *Arbacia* would represent nine cleavages (2^9).

[2] A very interesting study of these skeletons with regard to systematic relationships of the adults is given by v. Ubisch (1932).

PLATE III

White half, fertilized

Photographs 33–36. Elongate white half.
Photographs 37–53. White half after standing an hour after centrifuging.
Photograph 38. Monaster stage. Monaster does not show well, owing to lack of heavy granules.
Photograph 39. "Streak" stage. Streak does not show so well as in normal whole egg, but the enlarged nucleus is plainer.
Photograph 40. Nuclear membrane just broken.
Photograph 41. Amphiaster.
Photograph 43. Immediately after first cleavage; cells well separated. Cf. 22.
Photograph 44. Before second cleavage; cells close together. Cf. 23.
Photograph 49. Blurring caused by blastula swimming inside membrane.
Photograph 50. Hatching from fertilization membrane.
Photograph 51. Late blastula.
Photograph 52. Gastrula.
Photograph 53. Well-formed pluteus. Note lattice-like skeleton in arms. Cf. 32.

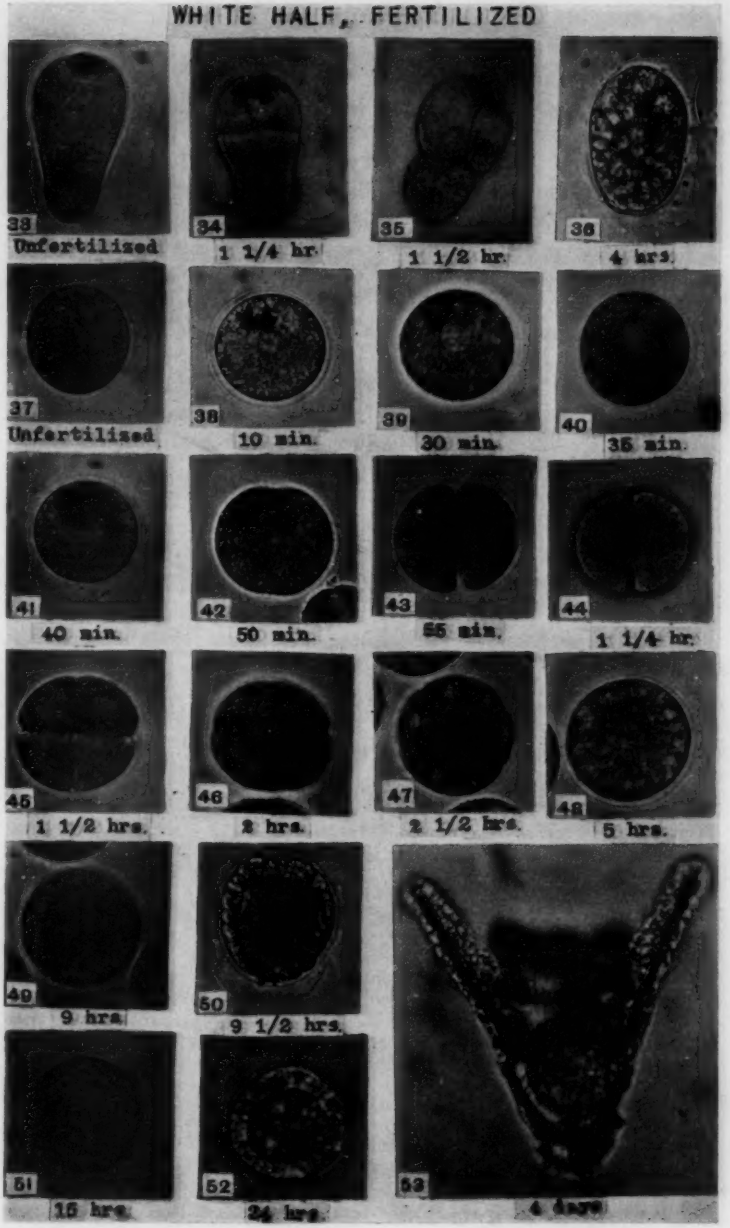
WHITE HALF, FERTILIZED
33
Unfertilized
34
1 1/4 hr.
35
1 1/2 hr.
36
4 hrs.
37
Unfertilized
38
10 min.
39
30 min.
40
35 min.
41
40 min.
42
50 min.
43
55 min.
44
1 1/4 hr.
45
1 1/2 hrs.
46
2 hrs.
47
2 1/2 hrs.
48
5 hrs.
49
9 hrs.
50
9 1/2 hrs.
51
15 hrs.
52
24 hrs.
53
4 days

PLATE III

White Half-egg (Plates III and IV)

The development of the white half-egg, made elongate by further centrifuging (20–30 minutes at 10,000 × g) and immediately fertilized, is shown in Photographs 33–36. It will be noted that the first cleavage plane goes across the short axis and divides the egg unequally (Photograph 34); it is usually in a position corresponding to that in the elongate whole egg (Photograph 13). The subsequent cleavages are similar in the two cases, and slipper-shaped white blastulae are formed (Photograph 36).

The development of the white half which has been allowed to stand for an hour or so till the granules are more evenly distributed, and then fertilized, is shown in Photographs 37–53. This half-egg lacks all the red pigment and most of the yolk granules and yet it cleaves and develops quite like the normal whole egg. After the first cleavage, the two cells are at first well separated and then come close together (Photographs 43, 44) as in the normal egg. Micromeres have not been observed, but they would be difficult to be sure of on account of the small size of the cells, and the lack of color contrast. Plutei have been raised, normal in every respect except for size and lack of pigment (Photograph 53). After a few days, pigment granules appear. The skeleton is of the lattice form typical of the pluteus from the whole egg.

Well-stratified spherical white halves as they occur immediately after having been centrifuged off from the whole egg (Photograph 54), will also develop when fertilized. The first cleavage plane comes in usually through the oil cap (Photograph 55), as it does in whole eggs which have been well stratified but not elongated; this was observed

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PLATE IV

White half fertilized, continued

Photograph 54. White half, immediately after removal from centrifuge.
Photograph 55. First cleavage through oil cap.
Photograph 56. First cleavage parallel with stratification.
Photograph 57. First cleavage diagonal.
Photographs 58, 59. White halves, together with whole eggs and red halves, fertilized, to show comparative development in same lot of eggs. Higher magnification of lot similar to 116.
Photographs 60–64. Abnormal development of white halves. Cf. Photographs 11–14 of 1940 paper.
Photograph 60. "Dauerblastula."
Photograph 61. Blastula with primitive triradiate spicule.
Photograph 62. Blastula with abnormal skeleton.
Photograph 63. Abnormal pluteus without arms.
Photograph 64. A group of abnormal plutei from one batch, together with one normal whole pluteus from the same batch of eggs.

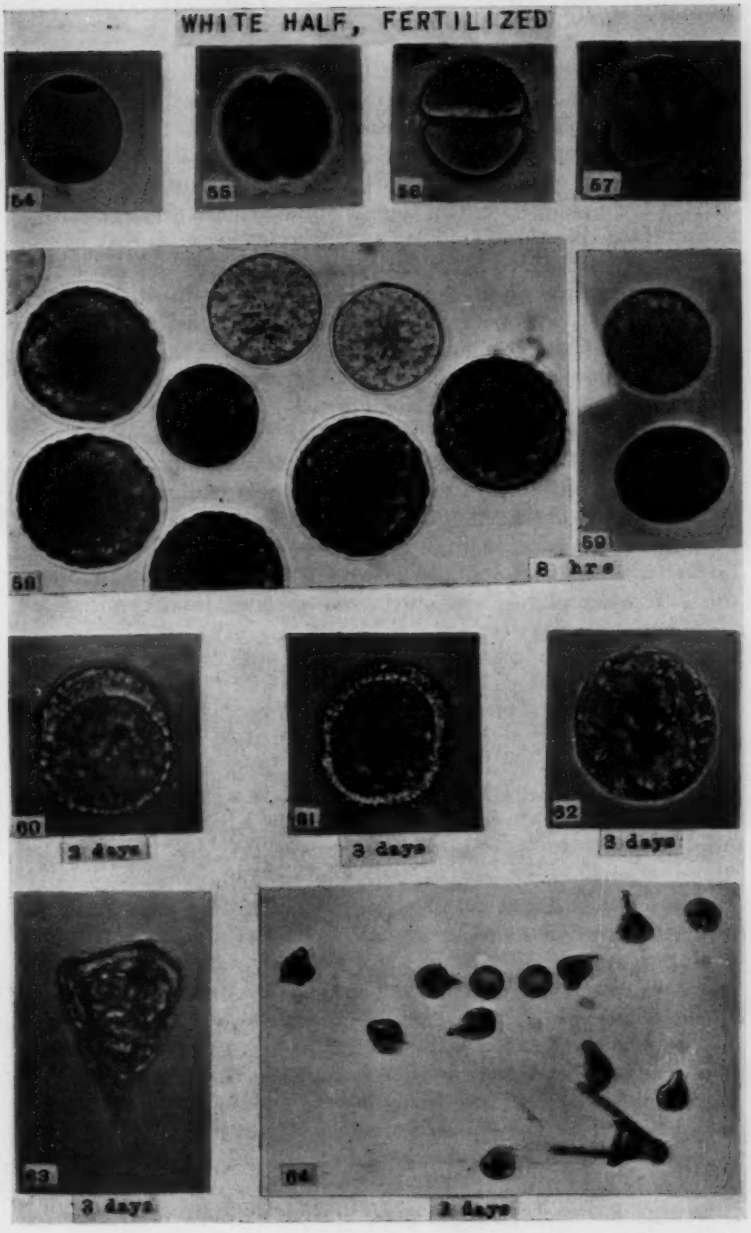
WHITE HALF, FERTILIZED
54
55
56
57
58
8 hrs
59
60
3 days
61
3 days
62
3 days
63
3 days
64
3 days

PLATE IV

for the whole egg in the first centrifuging experiments of Lyon (1907). He also observed that the first cleavage plane may come in, less frequently, parallel with the stratification, or at an angle. This is true also of the spherical white halves (Photographs 56–57).

Although many of the white halves develop into normal plutei, some develop abnormally. Among the abnormalities are permanent blastulae ("Dauerblastulae"), blastulae with primitive triradiate spicule or with imperfect skeleton, and abnormal plutei with skeleton and no arms (Photographs 60–64). In a recent paper (1940), it was shown that white halves obtained by centrifuging after fertilization may develop into abnormal blastulae and plutei quite similar to these, but in this case no normal plutei occur. Compare photographs 60–63 of this paper with 11–14 of the previous paper (1940). There seems to be no constant percentage of normal development in any one batch of white half-eggs, but certain whole batches develop much better than others, owing probably to better experimental conditions. In some batches, all are abnormal (Photograph 64; one normal pluteus from whole egg present for comparison).

The white half-egg will also develop parthenogenetically, and give rise to a white pluteus similar to that obtained from the fertilized white half.

Red Half-egg, Fertilized; Fertilized Merogone (Plate V)

The development of an elongate red half-egg, obtained by centrifuging a little longer (5 minutes at 10,000 × g) and in a slightly denser sugar solution, and immediately fertilized, is shown in Photographs 65–68. This egg has only the ♂ nucleus. The first cleavage plane is across the short axis and divides the egg unequally (Photograph 66), as in the elongate whole egg and white half. The following cleavages are likewise similar (Photographs 67, 68).

The development of the spherical red half is given in Photographs 69–88, and has been previously described (1932). The fertilization membrane and ectoplasmic layer are thicker than in the white halves. Fairly regular cleavages may take place, but with the pigmented cells usually larger (Photographs 73–77). A blastula, with small blastocoel is formed, and this emerges from the fertilization membrane in quite typical fashion and becomes free-swimming (Photographs 78, 79). Complete development into plutei is rare, and only a few normal or almost normal plutei have been obtained (Photograph 80).[3] In some cases, the fertilization membrane breaks during cleavage, and the cleavage cells spread out (Photographs 81–84). Also, the cleavage planes are apt to be omitted after nuclear division, so that multi-

[3] A number of absolutely perfect small plutei with lattice-like skeletons have recently (July 29) been obtained from fertilized red halves.

nucleate forms are common, or forms in which a few cleavage planes occur (usually in the lighter portion) and many nuclei in the uncleaved portion (Photographs 84–88). It seems likely that the great amount of heavy granular material interferes with the cleavage planes, and this may be responsible for the difficulty in raising these eggs to full development.

Red Half-egg, Parthenogenetic; Parthenogenetic Merogone (Plate VI)

The red half-eggs, though having no nucleus, can be activated artificially, by means of hypertonic sea water (see under "Material and Methods"), and they develop quite like the fertilized red halves, to a certain stage. They develop best if activated just after centrifuging, even if elongate. This may be due to the fact that the surface membrane is stretched and thinner and thus perhaps more permeable, so that the surface changes take place more readily. The development of the elongate egg is given in Photographs 89–92, and is similar to that of the elongate red half-egg, fertilized (Cf. Photographs 65–68).

The development of the spherical red half is shown in Photographs 93–112. The fertilization membrane and ectoplasmic layer are thick (Photograph 94) as in the fertilized red half. It will be noticed that a clear sphere is present a little later (Photograph 95), resembling the nucleus in the fertilized merogone; whether there is a definite membrane around it, is difficult to determine, though there seems to be a phase boundary. The monaster stage (Photograph 96) is common and striking, though the monaster is, in fertilized eggs, associated with the male nucleus which of course here is absent. Amphiasters are frequently seen and the cleavage plane may come in between the two asters in typical fashion (Photograph 97). The first cleavage plane may divide the egg equally, in any relation to the stratification, though more usually it is parallel with the stratification (Photographs 97, 98). Successive cleavages may be fairly regular, and a many-celled blastula formed (Photographs 99–101) exactly as in the fertilized red half (Cf. Photographs 73–77). This emerges in typical fashion from the fertilization membrane (Photograph 102), but has never developed into a pluteus. Some 500 cells have been counted in the cellular blastulae (Photograph 103), which is approximately the number of cells in a normal blastula at hatching (See p. 170, and footnote 1). There seems to be no blastocoel. The blastulae do not swim actively, though they move or are moved slightly. If they have cilia, they are short and irregularly distributed. Some of these organisms have lived for four weeks, and were still viable; they did not increase in size but were rather, smaller, and the pigment disappeared. (Photograph 104).

Just as in the fertilized merogones, the fertilization membrane frequently breaks during cleavage, and the cells become loosely arranged (Photographs 105–108; cf. 81–84). Also cleavage planes may be omitted, and multi-astral forms occur (Photographs 109–111), similar to the multi-nucleate forms of the fertilized merogone (Cf. Photographs 85–88). In both types, some of the cleavage planes come in and some are omitted, especially in the pigment portion (Photograph 108, right egg; cf. 84). In some batches of red halves, multi-astral forms occur spontaneously soon after activation. These are probably similar to the "artificial astrospheres" of Morgan (1896, 1899, 1900), and the "cytasters" of Wilson (1901) in parthenogenetic whole sea urchin eggs. In some batches there occur many blastulae in which very small clear spheres are observed, sometimes associated with the asters (Photographs 109, 111, 112). These certainly resemble small nuclei, and the similarity of these supposedly non-nucleate red halves to the nucleate (fertilized) ones is indeed striking (Cf. Photograph 112 with 88). These spheres may be re-formed nuclei without chromatin; the chromatin could hardly appear *de novo*, and no chromatin could be detected in stained preparations.

Many substances have been added to the sea water in an effort to obtain further development of the parthenogenetic merogones beyond the blastula. The substances were added in varying amounts before, after and during centrifugation, before and after activation. It was thought that possibly the substances might penetrate better while the

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PLATE V

Red half, fertilized (fertilized merogone)

Photographs 65–68. Elongate red half.
Photographs 69–88. Spherical red half.
Photograph 70. Soon after fertilization, to show fertilization membrane and thick ectoplasmic layer.
Photograph 71. Male nucleus.
Photograph 72. Monaster.
Photograph 73. Amphiaster. Cell division will come in perpendicular to stratification.
Photograph 74. Two-cell stage. Division has been parallel with stratification.
Photographs 73–77. Regular cleavages with cell division.
Photograph 78. Hatching from fertilization membrane.
Photograph 79. Free-swimming blastula.
Photograph 80. Almost normal pluteus.
Photographs 81–84. Less regular cleavages, with fertilization membrane ruptured so that cells are more scattered.
Photograph 84, right. Egg in which cell divisions have come in in light part, but not in pigmented part, though nuclear division has taken place.
Photographs 85–88. Nuclear division without cytoplasmic division. Notice that the nuclei are not uniform in size in any one egg. Many small nuclei are present in Photograph 88.

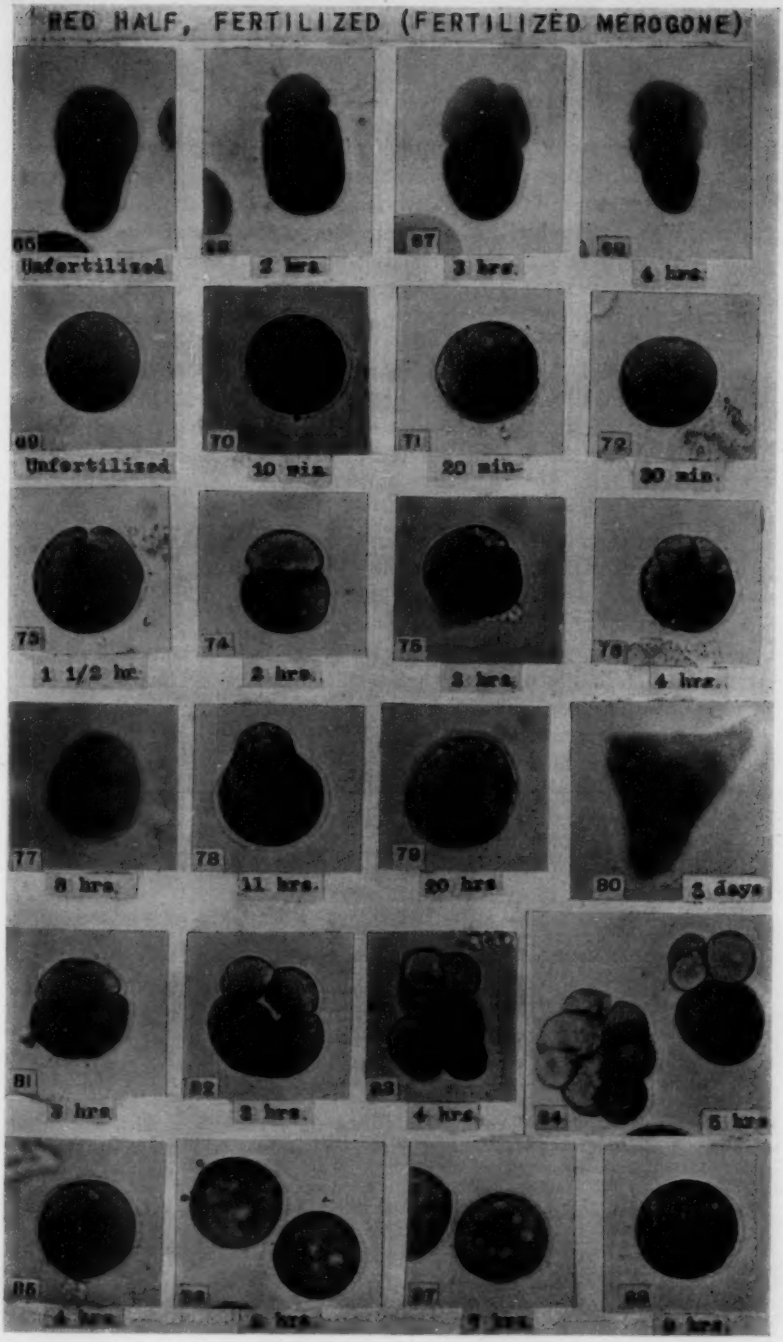
RED HALF, FERTILIZED (FERTILIZED MEROGONE)
Unfertilized
2 hrs.
3 hrs.
4 hrs.
Unfertilized
10 min.
20 min.
30 min.
1 1/2 hr.
2 hrs.
3 hrs.
4 hrs.
8 hrs.
11 hrs.
20 hrs
3 days
3 hrs.
3 hrs.
4 hrs.
5 hrs
4 hrs.
5 hrs.
7 hrs.
9 hrs.

PLATE V

membrane was stretched in centrifuging. Though some batches of eggs seemed to develop better than the controls in certain solutions, the results were never reproducible. Among the substances tried were materials associated with the nucleus, hormones, vitamines and dyes, as follows:—killed *Arbacia* sperm, living frog nuclei macerated, thymus nucleic acid, yeast nucleic acid, adenine, guanine, uracil, tobacco mozaic virus, *Megatherium* phage, adrenalin, pituitary, theelin, ascorbic acid, auxin, methylene blue, rhodamine, and also glutathione and leukotaxin. Variation of temperature, of concentration of the sea water, and different parthenogenetic agents were also tried. Preliminary experiments have been carried out of injecting some of these substances into the egg, but the technical difficulties are great.

Comparison of Shape, Rate and Size (Plate VII)

From the preceding paragraphs, it will be seen that the position of the cleavage plane differs with the shape of the egg. In spherical eggs, the first cleavage plane divides the egg equally. In elongate eggs, it passes usually through the short axis, parallel with the stratification, and divides the egg unequally. This is irrespective of the specific materials in the egg or half-egg, whether mitochondria, yolk or pigment. The position of the cleavage plane in elongate eggs seems to be determined rather by the relative consistency of the layers; it comes in where there are some granules, but not too many, and

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PLATE VI

Red half, parthenogenetic (parthenogenetic merogone)

Photographs 89–92. Elongate red half. Cf. 65–68.
Photographs 93–112. Spherical red half. Cf. 69–88.
Photograph 94. Soon after activation, to show fertilization membrane and thick ectoplasmic layer.
Photograph 95. Clear sphere simulating a nucleus. Cf. 71.
Photograph 96. Monaster.
Photograph 97. Amphiaster. Division will be perpendicular to stratification.
Photograph 98. Two-cell stage. Division has been parallel with stratification.
Photographs 97–101. Regular cleavages with cell division.
Photograph 101. Fine blastula with many cells. Cf. 77.
Photograph 102. Hatching from fertilization membrane. Cf. 78.
Photograph 103. Many-celled late blastula.
Photograph 104. Non-cellular parthenogenetic merogone, 4 weeks old.
Photographs 105–108. Cells somewhat scattered owing to rupture of fertilization membrane. Cleavages less regular and often asynchronous.
Photograph 106. Perfect 4-cell stage without membrane. Note asters.
Photograph 108, right. Egg in which cell divisions have come in in light part, but not in pigmented part. Cf. 84.
Photographs 109–111. Multi-astral eggs, with several small spheres associated with the asters. Cf. 85–87.
Photograph 112. Many small spheres resembling nuclei. Cf. 88.

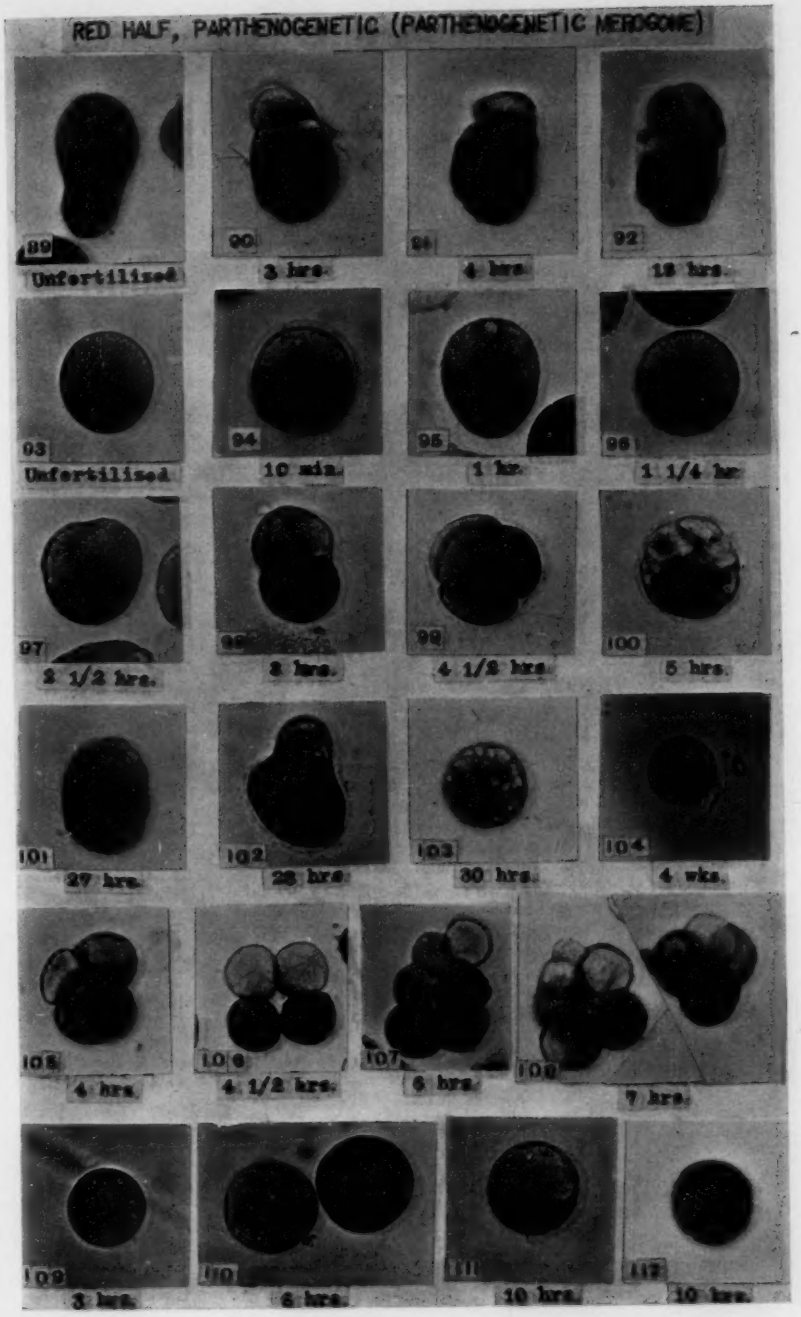
RED HALF, PARTHENOGENETIC (PARTHENOGENETIC MEROGONE)
89
Unfertilized
90
3 hrs.
91
4 hrs.
92
18 hrs.
93
Unfertilized
94
10 min.
95
1 hr.
96
1 1/4 hr.
97
2 1/2 hrs.
98
3 hrs.
99
4 1/2 hrs.
100
5 hrs.
101
29 hrs.
102
28 hrs.
103
30 hrs.
104
4 wks.
105
4 hrs.
106
4 1/2 hrs.
107
6 hrs.
108
7 hrs.
109
3 hrs.
110
6 hrs.
111
10 hrs.
112
10 hrs.

PLATE VI

usually not in the very narrowest part. The cleavage pattern does not, however, seem to affect final development.

The fertilized white halves cleave at the same rate as the whole eggs, if anything a little in advance (Photographs 113–116, 58, 59), and they hatch from the fertilization membrane at the same time. They are slower to differentiate; they are still blastulae when the whole eggs have become plutei (Photograph 117), and they become plutei the following day, but they are smaller than normal ones (Photograph 118). They are at first colorless, but acquire pigment later on. The parthenogenetic eggs, both whole ones and white halves, are slower in cleavage and development than the fertilized ones.

The fertilized red halves (fertilized merogones) cleave more slowly than the normal wholes and white halves. After 4 hours (Photograph 119), they are at about the same stage as the others at 2 hours (cf. Photograph 115). They hatch later, and differentiate still more slowly than the white halves. They are still blastulae after two days (Photograph 120), and become plutei on the following day (Photograph 121).

PLATE VII

Comparative

Photograph 113. Group of whole eggs, white and red halves to show comparative sizes.

Photograph 114. Similar group an hour after fertilization. The whole eggs and white halves are in the 2-cell stage, the red halves still uncleaved.

Photograph 115. Similar group a half hour later. The whole eggs and white halves are in the 2- and 4-cell stage, the red halves still uncleaved. The white halves are slightly in advance of the whole eggs.

Photograph 116. Similar group 7 hours after fertilization. The whole eggs and white halves are blastulae, the red halves much behind. The inset at right is a group of the whole eggs printed more lightly to show cleavage planes. A group similar to this, more highly magnified, is shown in Photographs 58, 59.

Photograph 117. The day after fertilization, the white halves are still spherical blastulae, the whole eggs are plutei.

Photograph 118. The following day, the white halves have become plutei; the small pluteus is from a white half, the others from whole eggs of the same age.

Photograph 119. A mixed group, printed lightly to show red halves, in 2- and 4-cell stages after 4 hours, similar to wholes and whites after 1½ hours. Cf. 115.

Photograph 120. The red half still a blastula after two days. A whole pluteus of same age alongside.

Photograph 121. The red half has become a pluteus the following day. A normal pluteus of the same age is alongside.

Photograph 122. A pure culture of parthenogenetic merogones, 4 hours after activation. Note the large numbers of cleaved eggs, in 2-, 3-, and 4-cell stages. The normal fertilized egg is many-celled at this time; cf. 27.

Photograph 123. A parthenogenetic merogone after three days, still a spherical blastula, of same size as previously. A normal pluteus of the same age is alongside.

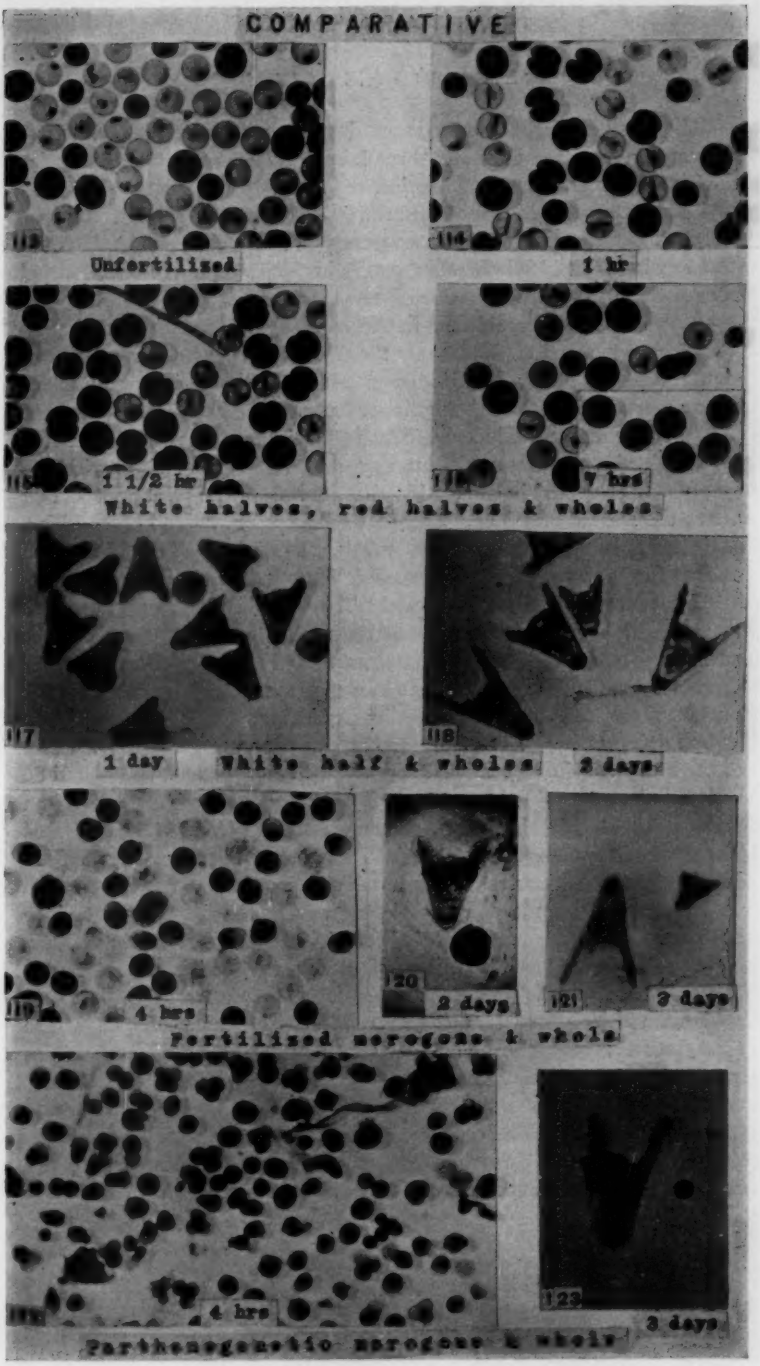
COMPARATIVE
113
Unfertilized
114
1 hr
115
1 1/2 hr
116
7 hrs
White halves, red halves & wholes
117
1 day
White half & wholes
118
2 days
119
4 hrs
120
2 days
121
3 days
Fertilized merogone & whole
122
4 hrs
123
3 days
Parthenogenetic merogone & whole

PLATE VII

The parthenogenetic merogones develop still more slowly. First cleavage takes place in about three hours, whereas it is two hours for the fertilized merogone, and 50 minutes for the white halves and wholes. In 4 hours they are a little behind the fertilized red halves (Photograph 122). The parthenogenetic merogones usually hatch only after 24 hours, whereas the whole eggs hatch in 9½ hours. And as stated above, they do not differentiate into plutei (Photograph 123).

As a general comparison of rate of cleavage, the eggs with two nuclei cleave more rapidly than those with one, whether parthenogenetic whole eggs or fertilized merogones. Those with one nucleus cleave more rapidly than those without any. The slow rate of cleavage of the clear quarters which have two nuclei is an exception (Harvey, 1932). Twice the amount of nuclear material in comparison with the cytoplasm, as occurs in the fertilized white halves, does not appreciably affect the rate of cleavage. But as we have seen, these halves differentiate, i.e. become plutei, more slowly. Possibly the lack of the full quota of cytoplasm may be responsible for the delay in differentiation.

The process of cell division and cell multiplication can go on with two nuclei, or one nucleus or none at all. Up to the present time, the parthenogenetic merogone has not gastrulated nor acquired a skeleton; practically no differentiation has taken place. And it may be that nuclear material is necessary for differentiation.

No attempt has been made to compare accurately the nuclear size of the half and whole eggs. A glance at Photographs 59, 74, 78 and 86 will show how variable is the size in a single egg. Also the nucleus in a normal egg changes in size before first cleavage from 11.5 μ to 16 μ. The nuclei in the clearer portions of the eggs are in general larger than in the pigmented portions in the same size cells.

No special granules seem to be necessary for development since both halves of the eggs, containing certain granules and lacking others, can develop into plutei. The red pigment seems to interfere with cell division but not with final development.

It should be stated that the times given for the various stages are by no means invariable. They depend upon the time of year as well as upon temperature and experimental conditions. This is especially true of the red halves which are very variable, both fertilized and parthenogenetic. Based on eight years of experience with the material, the times given are an average for development, as accurately as could be obtained, for a temperature of 23° C. at the height of the season.

Study of Stained Sections (Plate VIII)

Stratified Eggs (Photographs 124–127)

The eggs were fixed immediately after centrifuging, in Bouin's fluid, sectioned and stained with iron hematoxylin, and some were counter-stained with eosin and orange G. The clear layer of the whole egg (Photograph 124), which is optically empty in the living egg, stains blue, and is granular; this is apparently the protoplasmic ground substance, not moved by the centrifugal force used. This was described also in the early paper of Lyon (1907). The yolk stains orange with a rose tinge and the pigment orange. The mitochondria can sometimes be distinguished as a darker bluish band between the protoplasm and the yolk. The oil cap does not show; it is probably dissolved in the fixing or clearing fluid. The white half stains, of course, like the upper part of the whole egg (Photograph 125). The red half (Photograph 126) usually shows a blue cap of protoplasm, though this is obscured in the living egg by granules. In the red half further centrifuged till it is elongate, this cap of protoplasm formed by the further packing of the granules toward the heavy end, is larger (Photograph 127), and it is often seen in the living egg as a clear layer (Photograph 9).

Eggs Fertilized, Then Centrifuged (Photographs 128, 129)

When the eggs are fertilized and then centrifuged, the nucleus is always at the light pole, as in unfertilized eggs. At the stage after the nucleus has enlarged and the chromosomes have begun to form, just before the breaking of the nuclear membrane, the chromatin material is thrown to the heavy end of the nucleus as a dense mass (Photograph 128). This does not happen in the unfertilized egg (Photograph 124). The nucleolus of the immature egg is, however, thrown down to the centrifugal end of the germinal vesicle. If such fertilized eggs as mentioned above are left (living) in sea water after centrifuging, the material redistributes within 7 minutes, and normal spindle formation and cleavage follow.

When the fertilized eggs are centrifuged after the spindle has formed, it goes intact (with the forces used) to the light pole (Photograph 129) as found by Spooner (1911). There is no chromatin material left behind either before or after the nuclear wall has broken, as it would be perfectly visible in these stained preparations. There is no possibility, therefore, that the red halves contain chromatin material from the nucleus.

Chromosome Numbers (*Photographs 130, 131*)

The diploid chromosomes in the cleavage figures of fertilized eggs are small and crowded, and are very difficult to count with certainty, as other investigators have also found. The number is between 32 and 38 (Photograph 130). Tennent (1912) gives "about 40" for *Arbacia punctulata*, and Morgan (1927, p. 627) gives 38, quoting Wilson's data of 36–38. The number in the first cleavage of the parthenogenetic whole egg is approximately half the diploid. In Photograph 131, there are 16, but one or two may be missing.

The Mitotic Figure (*Photographs 132–140*)

In the early cleavages of the normal *Arbacia* egg, the spindle and asters are beautifully formed; the asters have rather thin rays. In Photographs 132–134 are given prophase, metaphase and anaphase figures. The mitotic figures of the fertilized white halves are similar to the normal egg. The fertilized merogone has also a spindle and

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PLATE VIII

Sections

(These photographs have been touched up to bring out details more clearly)

Photographs 124–127. Unfertilized eggs fixed in Bouin immediately after centrifuging, sectioned and stained in hematoxylin, eosin and orange G.

Photograph 124. Whole egg, showing protoplasm above, mitochondrial band, yolk and pigment. The oil cap does not show. Cf. living egg, Photograph 3.

Photograph 125. White half. Cf. living egg, Photograph 4.

Photograph 126. Red half; a protoplasmic layer is at the centripetal pole, not visible in living egg, Photograph 8.

Photograph 127. Red half, centrifuged further. Protoplasmic layer is greater; this is visible in a similar living egg due to greater packing of granules. Cf. Photograph 9.

Photograph 128. Fertilized egg centrifuged just before breakdown of nuclear membrane, showing chromatin material thrown down to heavy pole of nucleus. In the living egg, not centrifuged, this would be Photograph 18.

Photograph 129. Fertilized egg centrifuged at the metaphase, soon before cleavage. The spindle is intact at the centripetal pole. This is the stage shown in the living uncentrifuged egg in Photograph 20.

Photograph 130. Diploid group of chromosomes; 32–38.

Photograph 131. Haploid group from parthenogenetic whole egg; 16, one or two may be missing.

Photographs 132–134. Mitotic figure of normal fertilized egg. Prophase (132), metaphase (133) and anaphase (134). Forty to 45 minutes after fertilization.

Photographs 135–137. Mitotic figure of fertilized red half (fertilized merogone). Prophase (135), metaphase (136) and anaphase (137). Note slender spindle, and thick astral rays. One to 2 hours after fertilization.

Photographs 138–140. Mitotic (?) figures of the parthenogenetic merogone.

Photograph 138. Monaster. Note thick rays. One and ¼ hour after activation.

Photograph 139. Asters present in pairs in 2-cell stage. Note that those in the pigmented part have thicker rays than those in yolk. Four hours after activation.

Photograph 140. Two eggs overlapping, one in amphiaster stage before first cleavage, one in three-cell stage, each cell with a pair of asters. Six hours after activation.

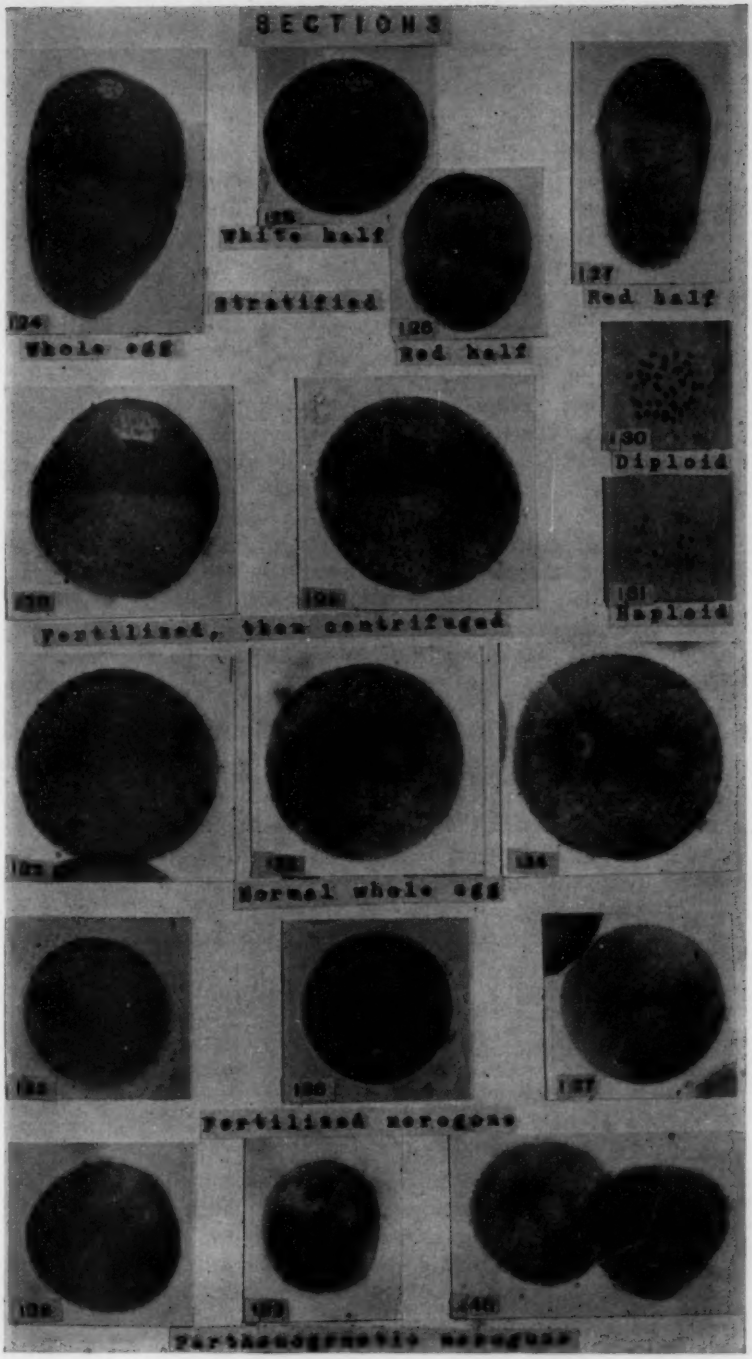
SECTIONS
124
Whole egg
125
White half
stratified
126
Red half
127
Red half
130
Diploid
131
Haploid
Fertilised, then centrifuged
Normal whole egg
Fertilised merogone
Parthenogenetic merogone

PLATE VIII

asters; the spindle is much thinner, often curved, and the rays of the asters are shorter and thicker; they are thicker in the pigmented than in the yolk portion. In Photographs 135–137 are given prophase, metaphase and telophase figures of the fertilized merogone. The parthenogenetic merogone has well-formed asters but no spindle; the rays of the asters are even heavier and thicker than in the fertilized merogone and thicker in the pigment area than in the yolk (Photographs 138–140). A large monaster is characteristic of the parthenogenetic merogone (Photograph 138), appearing in the same sequence of events as the monaster which develops in connection with the sperm in fertilized eggs. Later, two asters appear in the same sequence as the amphiaster in the fertilized eggs; the rays may approach each other toward the center, but there is never a well-formed spindle. In later cleavages, the asters may be found in pairs, also without spindles. In Photograph 40, there is one egg with an amphiaster and another egg, overlapping, with three cells, in each of which is a pair of asters. The original of the last photograph was sketched by four different investigators independently, and the sketches all agreed essentially. There is no question that asters are present, often in pairs, in the parthenogenetic merogones, but no spindle and no chromosomes. There is no special granule or centriole in any of the asters in the sea urchin egg.

Feulgen Reaction

The Feulgen reaction, which is specific for chromatin, is negative for the parthenogenetic merogones. Professor Jean Brachet very kindly helped me with the technique and examination of the eggs. The parthenogenetic merogones showed no red-staining material, whereas the fertilized merogones, prepared in the same way at the same time, showed it very clearly. There is apparently no chromatin material in the parthenogenetic merogones, at least in the early cleavages.

Summary

1. A comparative study has been made for *Arbacia punctulata*, especially by means of photographs, of the development of the normal whole egg, the white half, the red half fertilized (fertilized merogone) and the red half parthenogenetic (parthenogenetic merogone), all of these both spherical and elongate. The comparative rate of development is also given.

2. Development of the parthenogenetic merogones is not improved by applying various substances to the outside, such as nuclear compounds, hormones and vitamines.

3. A study of the stratification of the centrifuged egg and its halves as seen in prepared sections has been made.

4. Sections of eggs fertilized and then centrifuged show that at a certain stage, the chromatin material is thrown to the centrifugal pole of the nucleus, and that the spindle goes to the centripetal pole intact.

5. Cytological details have been studied, in sections, of the division figure of the normal egg, of the red half fertilized, and of the parthenogenetic merogone. During cleavage stages, the parthenogenetic merogone has well-formed asters, often in pairs, but no spindle and no chromosomes.

6. The Feulgen reaction is negative for the parthenogenetic merogones; there is no chromatin material.

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SOME EFFECTS OF COLCHICINE UPON THE FIRST CLEAVAGE IN ARBACIA PUNCTULATA[1]

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The pioneer work of Hertwig and Hertwig (1887), Boveri (1897), Wilson (1902) and others on the effects of various environmental conditions upon the division mechanism of cells has stimulated numerous workers to further study the effects of a wide variety of agents such as anaesthetics, poisons, injury, hypertonic and hypotonic solutions, centrifuging, mechanical agitation and various types of radiation upon this interesting process.

The recent advent of the use of colchicine (a narcotic alkaloid) to produce polyploidy in cells has led to numerous publications, mainly by cytogeneticists, on this general subject. However, few studies have been made in an effort to analyze the general effects of colchicine on the cell as a whole and particularly from the point of view of the mechanics of cell division. It was with these problems in mind that we undertook the present investigation.

Material and Methods

In preliminary experimentation it was found that 0.0002 molar concentration of colchicine in sea water when applied 10 minutes after fertilization was effective in preventing the formation of mitotic figures or cleavage furrows in the eggs of *Arbacia punctulata*. Furthermore, this solution was not lethal to the eggs as determined by their ability to undergo subsequent cleavage after being removed from the colchicine solution to sea water.

Experiments were therefore designed to determine at what stage or stages in the mitotic cycle the eggs could be prevented from cleaving by treatment with this concentration of colchicine. Accordingly, eggs were placed in the colchicine solution at two-minute intervals over a period of one hour after fertilization, at which time some of the eggs were examined for mitotic figures and cleavage furrows; some were fixed for a detailed study of the asters, spindle and chromosomes, and some were allowed to remain in the solution for 6 hours or longer to determine whether or not the cleavage mechanism was completely blocked or possibly only temporarily delayed by the treatment.

[1] Aided by grant from the Rockefeller Foundation for work on Cellular Biology.

To determine the effects of the colchicine upon the consistency ("viscosity") of the eggs, experiments were performed as follows: eggs were placed in the colchicine solution 10 minutes after fertilization and let stand 10 minutes. They were then centrifuged, together with controls, for the same time and at a speed just sufficient to produce a slight derangement but no extensive stratification of the control eggs. It is well known that the viscosity of the eggs varies considerably at different stages in the mitotic cycle. Therefore, untreated eggs of the same lot which were fertilized 10 minutes later than the experimental series were centrifuged for the same time and at the same speed. This was done in order to obviate the possibility that any difference in consistency between the experimental and control eggs might be due to a further development of the controls because of the possible blocking of the experimental ones by the colchicine solution.

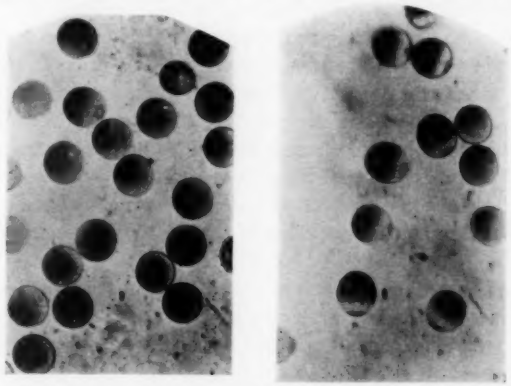

FIG. 1. Control eggs (left) and colchicine-treated eggs (right). Both were centrifuged 20 minutes after fertilization for the same time and speed. A comparison of the stratification indicates that a lowering of the viscosity has occurred in the colchicine-treated ones.

In each experiment only eggs from a single female and sperm from a single male were used. The eggs were kept in finger bowls at room temperature with the water varying from 22° to 24.4° C. Controls were run with each experiment.

Observations

All experiments in which the stratification of control and colchicine-treated eggs was compared after centrifuging showed the experimental eggs to have a lower viscosity as indicated by a more marked stratification of certain of their cytoplasmic components. The hyaline zone was only barely indicated in the controls, whereas it was well developed in the colchicine-treated eggs (Fig. 1). It is to be pointed out, however,

that the cortex of the experimental eggs did not in all instances show a complete displacement of their pigment granules, thus indicating a higher viscosity for the cortex than exists in the sub-cortical regions of the eggs. That this is true for the normally dividing eggs seems well established (Brown, 1934; Marsland, 1939). Higher centrifugal forces which produced definite hyaline areas in the control eggs also caused a derangement and some displacement of the pigment granules in the cortex of the colchicine-treated eggs, whereas in the cortex of the control eggs similar effects were not so striking. Centrifugal forces of the order of 15,000 to 20,000 times gravity readily displaced the pigment granules in the cortex of both the control and colchicine-treated eggs.

Fertilized eggs placed 10 minutes after fertilization in an 0.0002 molar concentration or stronger colchicine solution showed no mitotic figures or cleavage furrows developing. Weaker solutions of colchicine were observed to cause irregular cleavages, that is, the furrows grew in on the division plane from one side only. The mitotic apparatus in such cells was usually present but frequently showed evidence of being injured. Eggs that had undergone one division of the nucleus only in the colchicine solution when removed to normal sea water sometimes divided directly into 4 blastomeres or showed various types of abnormalities such as unequal division. Sections of such eggs frequently showed unequal separation of the chromosomes and multipolar spindles.

Fertilized eggs were allowed to stand 10 minutes after fertilization, at which time some were placed in a colchicine solution at two-minute intervals, extending over a period of 60 minutes. The eggs of all the samples were examined two to six hours later to determine the percentage that had undergone cleavage. The results of these experiments are given in Fig. 2.

It will be observed (Fig. 2) that no cleavages took place when the eggs had been allowed to develop not over 22 minutes (22°–24.4° C.) before being placed in the colchicine solution. However, if allowed to develop 28 to 36 minutes before being placed in the colchicine, at least 50 per cent of the eggs cleaved.

Cytological examination of the eggs placed in the colchicine solution 22 minutes after fertilization revealed them to be in the prophase stage of division. At 26 to 30 minutes after fertilization some were in late prophase, metaphase and anaphase stages of development. Attention should be called here to the work of Fry (1936) on the time schedule of mitotic changes in *Arbacia* eggs at Woods Hole. From his work, it will be observed that considerable variation occurs in speed

of development in *Arbacia* eggs even though they have all been taken from a single female. Furthermore, eggs taken from females at different months of the season were found to have different developmental tempos. Temperature is also a very important factor in the speed at which these eggs divide.

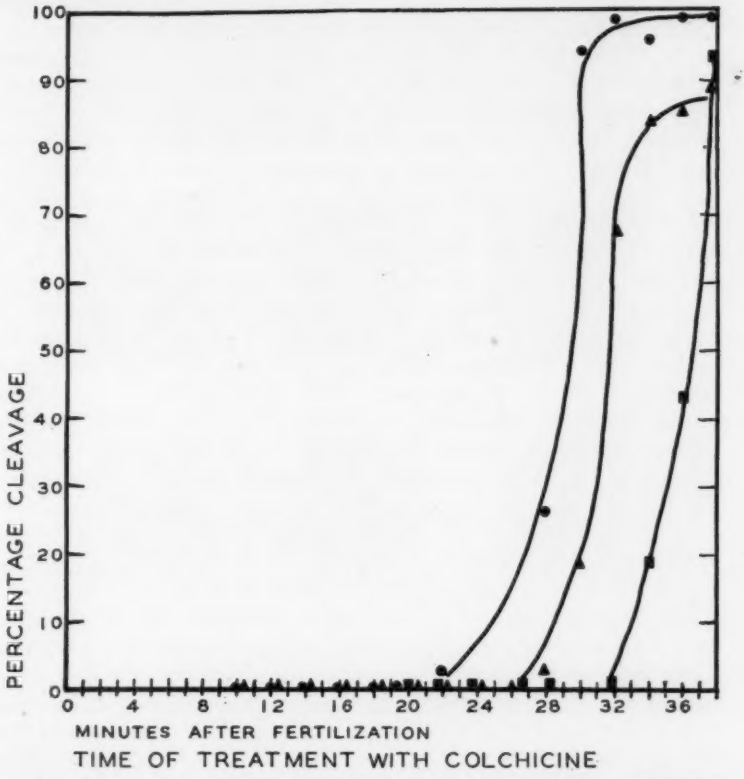

FIG. 2. A graph showing suppression of cleavage by colchicine. The results of three experiments are indicated by the curves. Note complete suppression until 22 minutes after fertilization. After this time the eggs rapidly became more resistant to the colchicine.

Dividing eggs that had been treated with colchicine were examined with a water immersion lens. The chief effect of the colchicine that could be detected by this method of study was upon the asters. As the colchicine penetrated the egg a gradual fading out of the astral rays occurred until eventually there appeared at each pole a clear

lake-like body. These, as we shall see later, represent the disintegration (liquefaction) of the asters.

In view of the fact that it is not possible to study accurately the chromosomes and mitotic apparatus in living sea urchin eggs, it was necessary to resort to fixed and stained preparations for this part of the study. Accordingly, we have recorded in Plate I some of the typical cytological changes that occurred following the colchicine treatment.

In Fig. 3 is illustrated a typical normal metaphase stage of division in *Arbacia*. It will be observed that the asters and chromosomes show clearly, while no indication of cleavage furrows is as yet to be observed. Figure 4 is a section through an egg taken from the same batch as that in Fig. 3 but placed in a colchicine solution at the same time that the one in Fig. 3 was fixed. After standing in the colchicine for 15 minutes the egg was fixed and stained in the same way as the egg in Fig. 3 had been. Here it will be observed that the asters have lost their rays, and have undergone an apparent liquefaction giving rise to two lake-like bodies in the cytoplasm. The chromosomes seem to be clumped, a condition that has been reported many times in various tissues following colchicine treatment. In this particular cell (Fig. 4) the cytoplasm appears to be more coarsely granular than in the normal egg but this is not a general characteristic of the colchicine effect, as may be noted by examination of Figs. 5 to 10. Figure 5 represents a section through an egg treated with colchicine while in the prophase stage of division. Here too the developing amphiaster has disintegrated, leaving a characteristic laked appearance in the cytoplasm. A much later stage in the mitotic cycle is illustrated in Fig. 6. In fact, cells advanced to this stage in the mitotic cycle sometimes cleave in spite of the colchicine treatment. However, here, as in the cases cited above, a disintegration of the amphiaster has taken place and the cleavage furrows have failed to form. On the other hand, Fig. 7 shows an egg from the same batch and treated in the same way as the one in Fig. 6 and apparently a normal separation of the chromosomes and cleavage has occurred. This might be explained by assuming that here a stage sufficiently late in the mitotic cycle had been reached where the asters had completed their chief function in the cleavage process before the colchicine was applied so that instead of blocking the cleavage mechanism, as occurs in the earlier stages of mitosis, it may have enhanced the process.

Various other types of cytological abnormalities following colchicine treatment will be seen in Figs. 8 to 10. In Fig. 8 two nuclei are seen which appear out of phase with each other so that the chromatin is not in the same physical state. The nucleus near the center of

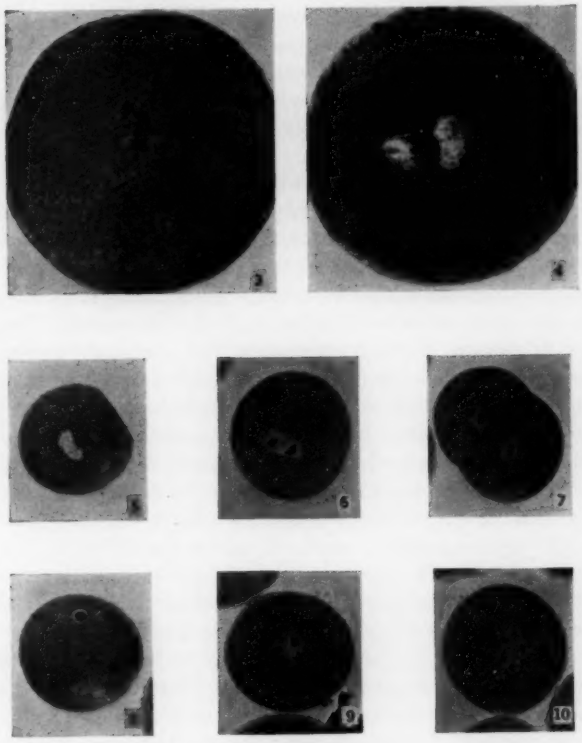

PLATE I

EXPLANATION OF FIGURES

All figures are photographs of *Arbacia* eggs fixed in Bouin's solution and stained in Heidenhain's hematoxylin. All except Fig. 3 have been treated with 0.0002 molar concentration of colchicine.

FIG. 3. Metaphase stage of control egg showing a typical mitotic figure.

FIG. 4. Egg in same stage as Fig. 3 when treated with colchicine. Note effects on the mitotic figure.

FIG. 5. Egg in prophase stage when treated with colchicine. A liquefaction of the spindle and asters have occurred as in Fig. 4.

FIG. 6. Later stage in the mitotic cycle showing destruction of asters and spindle after the chromosomes have separated. No cleavage furrows formed in this egg.

FIG. 7. Egg from same batch and treated in the same way as Fig. 6. Here the cleavage process occurred after the egg was placed in the colchicine solution.

FIGS. 8, 9 AND 10. Eggs showing various other types of abnormalities following colchicine treatment.

the cell seems to be in the early prophase; the one near the upper surface of the egg shows the chromatin clumped. Eggs that have been left in the colchicine solution for longer intervals have various abnormal groupings of their chromosomes, as indicated in Figs. 9 and 10, and also giant nuclei, an example of which is not shown in the plate.

Discussion

It is evident from the foregoing observations that the chief effect of colchicine upon the dividing eggs of *Arbacia* is to produce a lowering of the viscosity or at least a destruction of the gelation which normally occurs during division. This is accompaned by a rapid disintegration of the amphiaster and, when applied in the early stages of the mitotic cycle, an inhibition of the formation of the cleavage furrows. The chromosomes, however, may divide but usually they do not separate normally, thus producing either a restitution nucleus of a polyploid nature or several smaller nuclei in close proximity to each other. When the eggs are removed from the colchicine solution to sea water, many of the above-named changes are reversible.

Nebel and Ruttle (1938) have also mentioned the fact that colchicine in 10^{-4} molar concentration will block the cleavage process in *Arbacia punctulata*. In general our cytological observations upon the eggs following the colchicine treatment confirm theirs, but in their studies no efforts were made to correlate the cytological changes with the viscosity changes of the egg, or to determine at which particular stages in the mitotic cycle colchicine was effective in inhibiting the cleavage process.

Of the numerous agents (lipoid solvents, abnormal temperature, injury, drugs, hypertonic or hypotonic solutions, mechanical agitation, high hydrostatic pressure, etc.) that have been used to produce polyploidy in cells, none seems to be so effective and at the same time to produce so little harmful consequence to the cell as does colchicine. Because of this fact, it has been more successfully used by the geneticists to produce polyploidy in organisms than any other agent.

It is now definitely established from the works of Chambers (1917, 1924), Heilbrunn (1920, 1928), Fry and Parks (1934) and many others that in the course of cell division there is a marked physical-chemical change (viscosity) of the protoplasm associated with the different morphological changes (mitotic stages) in the cell. In fact, in the astral type of cleavage such as occurs in *Arbacia* the appearance of the asters and spindle seems to be an expression of a gelating action in the cytoplasm at these points which increases with the progressive development of the asters (Chambers, 1924; Heil-

brunn, 1928; Gray, 1931 and others). It is accordingly held by many that the asters are essential in the division mechanism of cells of this kind. This is substantiated by the fact that there are few, if any, authentic cases where cells that normally contain asters ever divide when these structures are absent or have been destroyed in the early stages of division.

The results recorded here substantiate this general view; in addition, an attempt has been made to determine at which stage in the mitotic cycle the amphiaster may be destroyed without blocking division of the eggs. In other words, is there a stage in the mitotic cycle before the appearance of the cleavage furrows in which the asters have completed their chief function in the division mechanism and after which time their rapid disintegration by colchicine will no longer completely block the formation of the cleavage furrows?

Our evidence seems to show that the critical point in mitosis, that is, the stage at which some of the eggs cleave and others were blocked when placed in the colchicine solution, occurred about 10 to 14 minutes before the appearance of the cleavage furrows of the controls when developing at temperatures between 22° and 24.4° C. Fixed, sectioned and stained eggs of this stage showed them to be in the late prophase, metaphase and a few in the early anaphase stages of division. It would seem then from this data that the abnormally rapid disintegration of the asters in *Arbacia* eggs once they have reached the late anaphase stage has little or no effect on the cleavage process. However, as pointed out above, destruction of the asters and the spindle in the prophase, metaphase or early anaphase blocks the cleavage process. It therefore appears that the asters and spindle are essential parts of the division mechanism of these eggs and that their main function in the process occurs in the early stages of the mitotic cycle.

It should be pointed out here that although the most generally accepted function of asters is that they play some kind of rôle in cell division, there are many animal cells which divide in the absence of demonstrable asters. However, whether or not a gel condition occurs at the poles of the spindles in such anastral mitosis has not been clearly demonstrated. Asters are usually found during mitosis in cells of large size although there are certain exceptions to this general rule. Fry (1934) has even suggested that they probably do not play any essential rôle in cellular activities.

The significant observations of Brown (1934) and of Marsland (1939) have demonstrated a marked variation in the viscosity of the cortex and interior of *Arbacia* eggs prior to and during cleavage. By means of high hydrostatic pressure they have been able to produce a

liquefaction of the cortex of dividing eggs so that no furrow forms and previously formed but incomplete furrows undergo recession. Preliminary observations seem to indicate that the amphiasters and spindle of such eggs were relatively unaffected by the pressure treatment.

To what extent the colchicine solution affected the viscosity of the surface of the egg was not so easily determined as was its effect upon the interior. However, our observations show that a slightly greater distortion and displacement of the pigment granules seemed to occur in the colchicine-treated eggs than in the controls. This would indicate at least a slight lowering of the viscosity of the surface of the egg, although it is not sufficiently great to inhibit the completion of the cleavage process once the furrows have started to form, differing here from effects of hydrostatic pressure. Furthermore, colchicine does not inhibit locomotion in *Amoeba* (unpublished observations) or cyclosis in the cells of *Tradescantia* (Nebel and Ruttle, 1938), both processes of which are thought by some to depend upon the sol $\rightleftarrows$ gel reactions at the surface.

Our work and that of others (Heilbrunn, 1915, 1920, 1928; Chambers, 1924, 1938; Brown, 1934; Gray, 1931; and Marsland, 1939) suggests that the division process in *Arbacia* eggs is associated with two separate but usually correlated processes (sol $\rightleftarrows$ gel reactions) occurring on the interior and at the surface of the eggs, the former being associated with the formation of the mitotic figure and the latter with the formation of the cleavage furrows. Experimental work has shown that the time of appearance of these reactions in the division process is of fundamental importance. A sol reversal of the mitotic figure by colchicine or mechanical agitation during the prophase or metaphase stage inhibits the formation of the cleavage furrows, while a sol reversal of the cortex at this time by mechanical agitation has no effect (Chambers, 1938). However, a few minutes before and during the formation of the cleavage furrows a reversal of the mitotic figure to the sol state by colchicine or by mechanical agitation has little or no effect on cleavage while a reversal of the cortex to a sol state at this time by mechanical agitation or hydrostatic pressure blocks the process (Chambers, 1938; Marsland, 1939).

The work with colchicine on dividing eggs seems to support the view that has been expressed by others, namely, that the spindle is essential for the proper separation of the chromosomes and that conditions which bring about a solation of the cytoplasm cause a destruction of the spindle while the chromosomes may be relatively unaffected.

Conclusions

1. Colchicine in concentrations of 0.0002 molar or stronger will inhibit cleavage in *Arbacia* eggs developing at 22° to 24.4° C. if applied before approximately 22 minutes after fertilization. Cytological observations have shown that eggs placed in the colchicine solution while in the prophase, metaphase or early anaphase stages are prevented from completing their cytoplasmic division.

2. The chief action of the colchicine upon the dividing eggs is to lower the viscosity or to inhibit and destroy the increased gelation that normally occurs within the eggs during division. Evidence for such action was determined by comparing under similar conditions the stratification of materials on the interior of control and colchicine-treated eggs. Colchicine also seems to lower slightly the consistency of the periphery of the eggs but not enough to cause a disintegration of the cleavage furrows once they have started to form.

Unpublished observations also show that colchicine does not inhibit locomotion in *Amoeba* which is thought to depend upon a sol $\rightleftarrows$ gel reaction at the surface, as is also the formation of cleavage furrows.

3. Colchicine produces a reversal of the asters and spindle to the sol state accompanied by a disappearance of the astral rays and spindle fibers. The chromosomes divide but do not separate normally due presumably to the loss of the directing force of the spindle.

4. The evidence obtained seems to substantiate the view that in the astral type of cleavage, such as occurs in *Arbacia*, the asters play an important part in the mechanism of cell division. Their destruction at any time before late anaphase seems to inhibit cleavage. However, in later stages of division an acceleration of the return of the asters to a sol state by colchicine does not inhibit the completion of the cleavage process. This indicates that the main rôle of the asters and spindle in the cleavage process occurs before the late stages in the mitotic cycle.

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DECOMPOSITION OF CHITIN BY MARINE BACTERIA

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Chitin is an important constituent of the exoskeletons of many marine animals. For example, the shell of the horseshoe crab, *Limulus polyphemus*, consists on an average of 25 per cent chitin. Due to the death of marine inhabitants, and to the production of chitinous casts during the developmental stages of certain species, large quantities of chitin are released annually. If this material were not decomposed, much carbon and nitrogen would be withheld from the cycles of these elements in the sea. Analyses of marine sediments indicate, however, that relatively little chitin accumulates there; it must, therefore, be broken down into simpler chemical compounds, or mineralized.

Decomposition of chitin by mixed or pure cultures of marine bacteria has been demonstrated by several investigators (2, 4, 12, 14, 16, 17), whose observations indicate that chitin-decomposing bacteria have a worldwide distribution in the sea. The present investigation was undertaken to study the distribution of these bacteria in a relatively restricted region of the sea, and to follow the breakdown of chitinous materials in natural media from this same locality.

Chitin has an elementary composition of $C_{32}H_{54}O_{21}N_4$ (6). It is a tough leathery substance, insoluble in water, in concentrated alkalis and in the usual organic solvents. By hydrolysis with concentrated acid it yields glucosamine and acetic acid in proportions which indicate that there is an acetyl group for each glucosamine residue. So far no essential differences have been found in the chitin from animal or vegetable sources (5, 7).

Isolation of Chitin-Decomposing Bacteria

Purified chitin, used in making culture media for the detection and isolation of chitin-decomposing bacteria, was prepared from horseshoe crab shells by the methods of Benton (1) and ZoBell and Rittenberg (17). The shells were treated successively, for prolonged periods, with 1 per cent hydrochloric acid, 2 per cent potassium hydroxide and 95 per cent ethyl alcohol. The snowy-white material which remained reacted positively to simple qualitative tests for chitin (3).

[1] Contribution No. 231.

Liquid culture media were prepared by partly covering 1 × 5 cm. strips of purified chitin, in test tubes, with sea water, or with sea water containing 0.1 per cent concentrations of additional nutrients such as dextrose, peptone, or ammonium chloride. In some cases a small amount (0.005 per cent) of phosphate was added to the sea water to eliminate any effect due to lack of available phosphorus. The medium best suited for the detection of chitin-decomposing bacteria consisted of a strip of chitin partly covered with a solution used by Waksman (14), and having the following composition:

Glucose	1 gram
Peptone	1 gram
K_2HPO_4	0.05 gram
Sea water (filtered)	1,000 ml.

Solid media were prepared from the liquid culture media either by the addition of 15 grams of agar to each liter of solution or by diffusion of the nutrient solutions through silica gels. Where solid media were employed strips of sterile purified chitin were placed on the surface of the hardened gel. The silica gel plates were prepared under as nearly aseptic conditions as possible; all other media were sterilized by autoclaving for 20 minutes at 120° C.

One additional solid medium was found useful for the rapid detection of chitin-decomposing bacteria. It was prepared by uniformly dispersing chitin in agar according to the following procedure. Fifteen grams of purified chitin were added to 150 ml. of 1 : 1 sulfuric acid and kept overnight in the refrigerator. The chitin was dissolved by this treatment but analyses showed that it was not altered chemically, i.e. hydrolyzed. The following day the chitin was precipitated by the addition of approximately 140 ml. of 20 per cent potassium hydroxide. The precipitate was placed on a Büchner funnel and washed repeatedly with distilled water until the filtrate was neutral to litmus. Thirty-five grams of the chitin, as removed from the filter, were then added to agar which contained 0.1 per cent glucose, 0.1 per cent peptone, and 0.005 per cent dipotassium hydrogen phosphate. After autoclaving, the agar was thoroughly shaken to redistribute the chitin, and Petri plates were prepared. In this condition the medium was of a uniform milky-white opacity. When chitin-decomposing bacteria grew on the surface of this agar they dissolved the chitin, thereby producing a clear halo around each colony. By noting the length of time necessary for the appearance of halos the rates of bacterial activity could be estimated. Bacteria which decomposed chitin rapidly often produced a halo after two days' growth whereas others required eight to ten days for a positive test. Although bacteria which were

unable to decompose chitin often grew well on the medium, they did not alter it in appearance. Results obtained with this agar confirmed, without exception, the results obtained with other solid and liquid media.

In order to test a given sample of marine material for chitin-decomposing bacteria, several different media were inoculated. The inoculated media were incubated at room temperature and examined periodically for evidence of chitin breakdown. Growth was slow in the medium consisting of only chitin in sea water, whereas the same bacteria decomposed the chitin more rapidly when additional nutrients were present.

Visible dissolution of the chitin, either in liquid medium or on a solid agar surface, was indisputable evidence that the chitin was being attacked. To detect incipient decomposition a hand lens was helpful. The liberation of ammonia from media lacking peptone or nitrate, reducing substances from media lacking glucose, or a pronounced change in acidity, were further reliable evidence of chitin decomposition. The mere growth of bacteria in a medium consisting of only chitin in sea water is an unreliable criterion of decomposition, since bacteria may persist in this medium for a long period without altering the chitin.

In preliminary studies, cultures of bacteria showing no evidence of chitin-decomposition were kept one year before being discarded as negative. It became evident, however, that several weeks were adequate for detecting the chitin-decomposing capacity of most species. To obtain pure cultures of bacteria a second tube of enriched medium was inoculated with a loopful of material from an original positive culture. Growth from this tube was then streaked on nutrient agar plates and the different types of colonies which developed were retested for their ability to attack chitin. In later experiments pure cultures were obtained more quickly by streaking growth from enriched cultures on agar medium containing precipitated chitin. This medium was highly satisfactory for separating the chitin-decomposing bacteria from others which had persisted but which were not attacking the chitin.

Occurrence of Chitin-Decomposing Bacteria in the Sea

The number of chitin-decomposing bacteria in sand, mud, and water was determined by dilution culture methods. To increase the probability of detecting chitin-decomposing bacteria, several kinds of culture media were inoculated with each dilution of the sample. In making quantitative determinations only visible breakdown of the chitin was considered a positive test.

An 11 cm. core of sand, taken from a littoral zone in the vicinity of Woods Hole harbor on July 31, 1939, was found to be a relatively rich source of chitin-decomposing bacteria. At the top of the core there were at least 60,000 of these bacteria per gram of sand, this number decreasing to 6,000 per gram at a depth of 5 cm., and to 600 per gram at 11 cm.

A sample of mud was collected on July 7, 1939, at latitude 40° 38′ N. × 71° 39′ W. and at a depth of 878 meters. Chitin-decomposing bacteria were relatively abundant in the upper layer of the core (125 cells per gram), but dropped to less than 5 cells per gram at depths of 5 and 11 cm. Both in sand and in mud the number of chitin-decomposing bacteria decreased sharply with core depth.

On August 2, 1939, a sample of water was collected at 5 fathoms NE. of Lambert's Cove, Martha's Vineyard, one mile offshore, in a locality where the water is naturally well stirred. Dilution culture methods indicated the presence of 150 bacteria per ml. of water.

Chitin-decomposing bacteria were isolated from other samples of sand, mud and water, in addition to those described above. There was no correlation noted between abundance of the bacteria in the sediments and depth of the overlying water. In addition to bacteria isolated from media collected in the vicinity of Woods Hole a few cultures of chitin-decomposing bacteria were isolated from samples of water brought from tropical regions by the research ship "Atlantis."

Dead and decomposing horseshoe crabs offered an excellent source of chitin-decomposing bacteria. A single attempt to isolate these bacteria from a jar of dead copepods was unsuccessful.

That animals may possess intestinal fauna and flora capable of breaking down certain foods ingested by the host is well established. It has been demonstrated, for example, that wood-boring termites harbor in their intestinal tract microörganisms which perform for their host the invaluable function of digesting cellulose (15). Since a similar condition in relation to chitin might exist in marine organisms, the intestinal contents of several common marine animals were examined for chitin-decomposing bacteria. Using methods already described for their detection, chitin-decomposing bacteria were found in the intestinal contents of *Venus mercenaria mercenaria*, *Ostrea virginica*, *Loligo pealeii pealeii*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus*, *Raia erinacea* and *Spheroides maculatus*. In this investigation, all animals whose intestinal contents were examined for chitin-decomposing bacteria were found to possess them. The presence of these bacteria in the intestines of marine animals has previously been demonstrated by Benton (1), who isolated them from the speckled

trout and by ZoBell and Rittenberg (17), who found from one hundred to more than a thousand chitin digesters in each milliliter of the stomach contents of the squid.

There are few marine animals which do not ingest chitin if they come in contact with it. According to available information (8, 9, 10), however, the oyster does not take in chitin at any time. Accordingly, it is interesting to note that chitin-decomposing bacteria were found in the alimentary canal of this animal, also.

Decomposition of Limulus Shell in Natural Media

Having demonstrated the presence of chitin-decomposing bacteria in marine habitats, it was decided to follow the breakdown of a naturally-occurring chitinous material in several marine media. Accordingly, *Limulus* shells were scrubbed free of flesh, cut into strips approximately 3 × 8 cm., weighed (about 3 grams after drying overnight at 100–105° C.) and placed in marine sand, mud and sea water. Sand from the littoral region was scooped up, to a depth of 15 cm., and thoroughly mixed. Weighed strips of crab shell were placed in glass jars and covered with 200 grams of the sand. Likewise, strips of *Limulus* shell were covered with bottom mud collected in the Woods Hole harbor. Sea water, collected a few feet from the shore, was placed in bottles in 200 ml. volumes. Weighed strips of crab shell extended about 1 cm. above the water level. All cultures were kept in a dark cabinet at room temperature. Occasionally distilled water was added to the sand and mud cultures to prevent them from becoming dry.

These experiments were continued for 57 weeks. At stated intervals one culture from each of the three series (sand, mud, water) was examined. The crab shell was removed, weighed and checked for visible evidence of decomposition. Any changes in color and texture of the natural media were noted. The sand and the mud were analyzed for total organic carbon and nitrogen. The carbon was determined by Tiurin's modification (13) of Schollenberger's method (11). The nitrogen determinations were made by the usual Kjeldahl procedure. The results of the analyses are based upon the total dry sand or mud (dried overnight at 100–105° C.) not freed from the sea salt.

In sand, decomposition of the *Limulus* shell began at once. At the end of one year, when the experiment was discontinued, the shell had lost about three-fourths of its original weight (Fig. 1) and had been reduced to a small fraction of its former size. At the beginning of this experiment the sand contained 0.8 per cent carbon, and 0.05 per cent nitrogen, indicating that its organic content was relatively low. Al-

though a large amount of organic material was decomposed during the course of a year, there was no measurable increase in carbon and nitrogen in the sand. Presumably the compounds liberated from the crab shell were used in the metabolism of the bacteria.

When *Limulus* shell was placed in bottom mud, decomposition of the shell, as measured by decrease in weight, was detectable only after a period of about six weeks. Thereafter the shell decomposed fairly rapidly until at the end of one year it had lost a third of its weight (Fig. 1). The original mud was high in organic content. When brought into the laboratory it contained 2.4 per cent carbon and 0.17 per cent nitrogen. During the course of decomposition of the crab

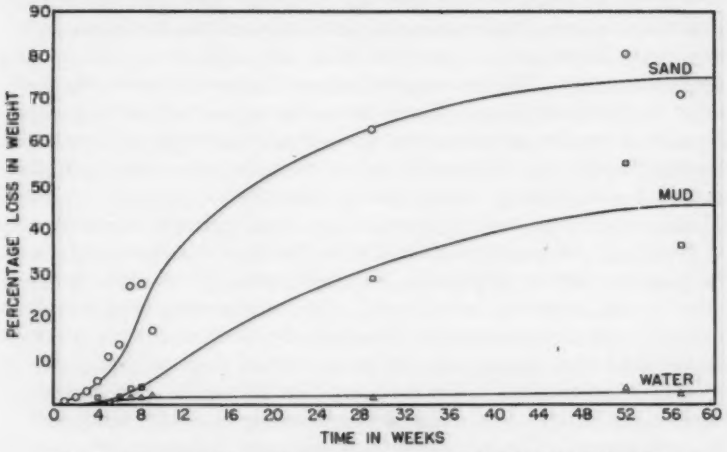

FIG. 1. Decomposition of horseshoe crab shell in natural marine media.

shell over a period of one year, the amount of carbon in the mud remained unchanged, while the nitrogen increased from 0.17 to 0.22 per cent.

In sea water there was only a very slight decrease in weight of the crab shell, even after a period of more than a year (Fig. 1). At the end of this time the shell was practically unchanged in appearance. This need not mean that chitin-decomposing bacteria were absent from the sea water, but only that under the experimental conditions there was no marked breakdown of the chitin in this medium, whereas in sand and in mud, under comparable conditions, the decomposition was pronounced. As far as laboratory experiments can show, chitin appears to be decomposed more slowly by marine bacteria than are most other structural organic materials.

During decomposition the shell underwent striking physical changes. Originally tough and leathery, it became soft and fragile as large areas were removed. Incipient decomposition was detected by using a binocular microscope, when the shell was observed to be "eaten" away in spots. In advanced stages of decomposition the shell was greatly reduced in size as well as in weight. In preparing purified chitin from *Limulus*, it was noted that continuous sheets of the purified material were obtained from all parts of the shell. It follows, therefore, that as the latter decomposed in these natural marine media, the chitin was digested along with the other compounds which make up the horseshoe crab shell.

The detailed characteristics of these bacteria are now being investigated. Preliminary tests indicate that approximately fifteen types of bacteria have been isolated. They are Gram negative, asporogenous, motile rods. Among the readily demonstrable products resulting from the decomposition of purified chitin by pure cultures of the bacteria are ammonia, reducing substances, and organic acids.

Summary

Chitin-decomposing bacteria were isolated from the following sources: marine sand, mud, water, decomposing horseshoe crabs, and the intestinal contents of *Venus mercenaria mercenaria*, *Ostrea virginica*, *Loligo pealeii pealeii*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus*, *Raia erinacea* and *Spheroides maculatus*.

Chitin-decomposing bacteria were found to be most abundant in marine sand, less abundant in mud, and present in small numbers in sea water.

Under experimental conditions, the decomposition of *Limulus* shell was relatively rapid in littoral sand, moderate in bottom mud, and negligible in sea water.

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THE EFFECTS OF IMPLANTING ADULT HYPOPHYSES INTO SEXUALLY IMMATURE FUNDULUS

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The factors which control the sex cycle of vertebrates are becoming increasingly clear as a result of numerous investigations. There is gratifying agreement, among all workers on animals above fishes in the evolutionary scale, that the pituitary gland plays a dominant rôle in the direct control of periodic changes that occur in the gonads and associated sexual organs. Control of the sexual cycle in fishes, however, is little understood. Efforts to determine the rôle which the pituitary gland may play in controlling this cycle have yielded conflicting results, particularly with reference to the effect of injecting mammalian pituitary extracts or pituitary-like preparations. Thus Calvet (1932) and Damas (1933) succeeded in stimulating the ovaries of lampreys with prolan and extracts of the mammalian pituitary gland; Boucher, Boucher and Fontaine (1934) obtained an enlargement of the gonads of both sexes in eels which were injected with pregnancy urine and saw evidence of maturation of sperm in the testis; and Morosowa (1936) found that when either pregnancy urine or prolan was injected into perch, laying of eggs and ejaculation of sperm could be produced at any time during the winter. On the contrary Koch and Scheuring (1936) using prolan, Matthews (1939) with injections of sheep pituitary extract and Johnson and Riddle (1939) employing three different mammalian extracts, all of which were shown to be active in stimulating the gonads of birds and mammals, obtained no such effect when these materials were injected into fishes. Fairly uniform results have been obtained, however, by injecting fish pituitary preparations into other fishes. Houssay (1930, 1931), Cardoso (1934), Pereira and Cardoso (1934), von Ihering (1935), and Azevedo, Dias and Vieira (1938) have all observed that the injection of saline suspensions or extracts of fish pituitary glands into other fishes resulted in an enlargement of the gonads and frequently in expulsion of eggs and sperm as well. In the work of von Ihering and of Azevedo, Dias and Vieira mating activities were also elicited in this way. More recently

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Hasler, Meyer, and Field (1939) found that when pituitary extracts of carp were injected into trout, mature eggs and sperm were produced six to seven weeks before the normal breeding season. Further evidence that the pituitary gland may be of importance in controlling changes in the gonads of fishes has been obtained by removing the gland. Vivien (1938, 1939) found that after removal of the hypophysis from *Gobius* marked regressive changes occurred in both testis and ovary; and Matthews (1939) showed that when male *Fundulus* were hypophysectomized and kept until the next breeding season the testes did not enlarge and become active as did those of control animals, and that no sperm and very little spermatogenic activity were present in the operated animals.

Work so far carried out on fishes, then, indicates that the fish pituitary gland contains some material capable of modifying the normal sex cycle, and suggests that in these forms, as in other vertebrates, the hypophysis is of importance in controlling changes in the gonads and associated organs. If this be true, then the injection of teleost pituitary extracts or the implantation of fresh preparations might be expected to produce an effect on the gonads of sexually immature animals, stimulating them to develop earlier than they normally would. The following is a report of experiments carried out on sexually immature individuals of *Fundulus* to determine whether or not precocious development of the gonads could be produced in this way.

An adequate supply of small *Fundulus* was collected in the small salt water streams on Penzance Point, at Woods Hole, by means of a small trap. A number from each group collected were killed immediately and their gonads examined. Of 136 animals examined in this way, ranging in length from 22 to 40 mm. from the tip of the snout to the base of the caudal fin, only 4 of the 65 males (lengths 32, 34, 39 and 40 mm.) showed whitish, active testes with sperm in fresh smear preparations, characteristics of the adult male during the breeding season. The remaining 61 males had testes that were definitely inactive. All 71 of the young females showed only small ovaries with no large follicles. Obviously the vast majority of these small animals were sexually immature.

Attempts were made to stimulate the gonads of these immature animals in two ways. In the first series of experiments the pituitary gland of a sexually mature *Fundulus* was implanted into the eye of an immature animal. From the success achieved with this method in mammals it was hoped that the transplanted gland would maintain itself and provide a continuous supply of any hormones produced by it to the host. From July 25th to August 5th 95 such implants were

made. The animals were kept in tanks provided with running sea water and a number of untreated young animals were placed in adjacent tanks for controls. Both groups were fed two or three times a week with fresh clam meat. Three experimental animals with controls of the same lengths were killed, two four weeks after implantation of the adult hypophysis and one five weeks following this operation. Of two experimental females only one showed a marked enlargement of the ovary over those of the controls, but microscopic sections showed that in both animals the ovarian follicles and their contained eggs were much larger than those of the control ovaries and that they were provided with larger amounts of vesicular yolk. The testis of the implanted male was also large, was whitish in color, and showed many sperm both in fresh smears and in microscopic sections; while that of the control male, like that found in the majority of untreated males of this size, was small, practically colorless and showed no sperm. The implanted pituitaries were recovered from the male and from one of the females. Sections of them showed fairly normal cells. In only one of them (that of the male) could blood vessels be traced from the tissues of the eye to the implanted gland.

The remaining animals were lost in an attempt to transport them from Woods Hole to Williamstown at the end of the summer.

The following summer a second series of the same sort was run. During the last week in July implants of adult hypophyses were made into the right eyes of 185 young animals, with 112 others serving as controls. For some undetermined reason there was a high mortality in this series. Only 12 males and 12 females of the experimental animals and 38 males and 37 females of the controls were still alive during the first week in September when they were killed and examined. In the females no effect of the implants on the ovary was observed in any case. Of the experimental males only 1 animal, 32 mm. in length, showed an active testis. The implanted pituitary glands were recovered from a number of these animals. Differences between them and the glands recovered from the previous series, where a certain amount of stimulation of the gonads had apparently occurred, were not striking, although in this latter series the cells were somewhat more shrunken and fewer granules were present in the cytoplasm.

Thus of the 27 young animals examined after implanting an adult pituitary gland into the eye, increased activity of the gonads over those of controls was apparent in only four. Since untreated animals of this size occasionally show similar development of the gonads (as in the four males with active testes mentioned earlier), it was certainly not

clear that the observed increase in activity of the gonads of these 4 experimental animals had been produced as a result of the implantation of the adult gland. In fact, the failure of the other 23 cases to show any effects suggested rather that the implanted glands lacked effective sex-stimulating hormones. It was possible, however, that the transplanted glands did not establish themselves successfully in the eye and that as a result such hormones as they might normally produce were delivered to the circulation for too short a time to be effective. In order to determine which of these possibilities was correct a second method of implanting the adult gland was employed. Preliminary experiments showed that when the pituitary gland of an adult was placed in the peritoneal cavity of a sexually immature animal through a small slit in the body wall the gland could be recovered in an apparently normal condition four or five days later. Twenty-seven young animals were treated in this way. Since the sex of the young animals could not be distinguished, no attempt was made to select the sex of the donors. As in the previous series the experimental animals and controls of appropriate sizes were kept in running sea water in adjacent tanks, both being fed twice a week with fresh clam meat. At three-day intervals each of the experimental animals received a fresh pituitary gland from an adult animal. Such serial implants should provide the immature animal with a fairly constant supply of whatever hormones might be liberated by the adult gland. These animals withstood the handling necessitated by the operations very well. The series was begun on August 8th and ran until September 3rd, during which time only three of the experimental animals died. Records were thus obtained on 24 animals, 10 females and 14 males. In every case the gonads of the experimental animals were much larger and more mature than those of the controls. The females showed not only an increase in size of the whole ovary (Fig. 1) but a marked increase in size and maturity of the eggs (Figs. 2 and 3). The effects on the males were even more marked. The testes of all experimental animals were white and very active, with a high degree of spermatogenic activity as shown in microscopic sections (Figs. 4, 5 and 6). Moreover, in these experimental males the dorsal fin had a prominent black pigment spot which several workers (Newman, 1908; Chidester, 1917; Parker and Brower, 1935) have shown is associated with sexual activity in adult males (Fig. 7).

From these experiments it is clear that when pituitary glands of sexually mature *Fundulus* are implanted into an immature animal in such a way as to provide a continuous supply of fresh gland material, pronounced stimulation of the gonads of both sexes is produced. The

evidence obtained from these experiments coupled with that previously obtained by removing the gland in adult males indicates that the pituitary gland of *Fundulus*, like that of vertebrates above fishes in the evolutionary scale, contains a sex-stimulating substance, presumably a hormone, which is of importance in controlling the development and subsequent seasonal changes in the gonad of this teleost.

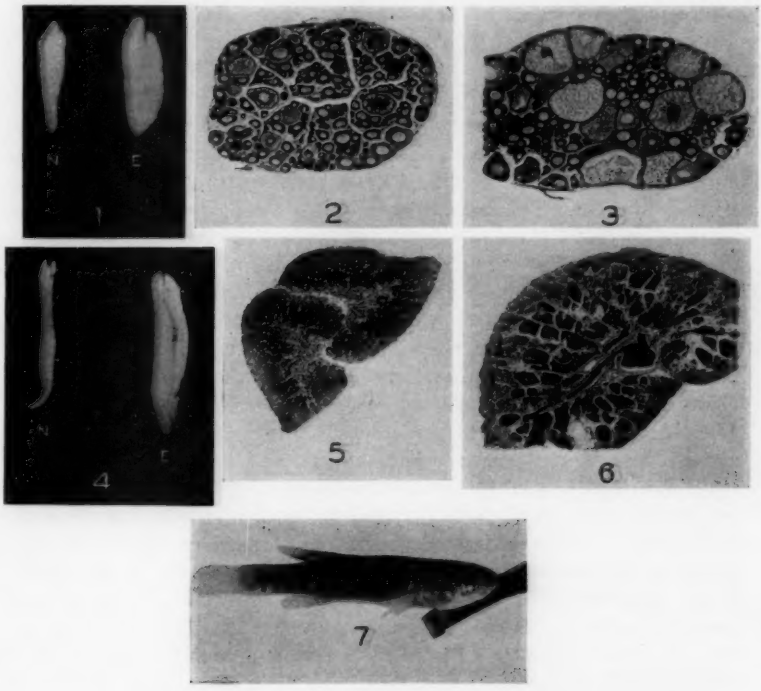

FIG. 1. Ovaries of young *Fundulus*, ×2.5. *N*, ovary of normal animal, 32 mm. long; *E*, ovary of female of same length which had received six adult pituitary glands over a period of 26 days.

FIG. 2. Cross-section of normal ovary shown in Fig. 1, ×25.

FIG. 3. Cross-section of experimental ovary shown in Fig. 1, ×25.

FIG. 4. Testes of young *Fundulus*, ×2.5. *N*, testis of normal animal, 37 mm. long; *E*, testis of male of same length which had received six adult pituitary glands over a period of 26 days.

FIG. 5. Cross-section of normal testis shown in Fig. 4, ×45.

FIG. 6. Cross-section of experimental testis shown in Fig. 4, ×45.

FIG. 7. Experimental male, 23 mm. long, in which the testis had been activated by implanting adult pituitary glands. Note pigment spot on dorsal fin.

That this is true of other teleosts as well is indicated by the work on other forms already reviewed. The evidence is quite clear that the teleost pituitary gland, when suitably injected or implanted, will stimulate activity in the gonads of other fishes. While the evidence obtained from injecting mammalian pituitary preparations is not as clear because of conflicting results, certainly most of the recent work shows that such mammalian preparations are either relatively or completely ineffective in producing gonadal stimulation in fishes. These facts suggest that a certain degree of zoölogical specificity of the pituitary hormones may exist, a point of view which has recently been discussed at some length by Creaser and Gorbman (1939). In this connection it is of interest to note that mammalian sexual hormones of both sexes, unlike mammalian pituitary preparations, have produced quite consistent results. Thus Fleischman and Kann (1932) showed that the injection of progynon into adult *Rhodeus* during the inactive period of the sexual cycle led to development of the oviduct comparable to that found during the breeding season; Berkowitz (1937, 1938) also showed that the feeding of progynon tablets to *Lebistes*, beginning at birth, caused a suppression of male secondary sexual characters and a marked suppression of spermatogenic activity, without, however, producing any effect on the secondary sexual characters of the female; and Eversole (1939) showed that the injection of testosterone propionate into *Lebistes* suppressed both body growth and ovogenesis in the female and induced the development of some male secondary sexual characters; while Regnier (1939) caused male secondary sexual characters to appear in females of both *Lebistes* and *Xiphophorus* and produced degenerative changes in the ovary by injecting testosterone propionate or merely by adding it to the water of the aquarium in which the animals were kept. Whatever factors are responsible for the lack of response met with when mammalian pituitary preparations were applied to fishes apparently do not apply to mammalian estrogenic and androgenic substances.

Summary

Pituitary glands of adult *Fundulus* were implanted into the eyes of sexually immature animals. Of twenty-seven cases examined several weeks after operation the gonads of only four showed greater activity than did those of control animals.

In another series of experiments adult pituitary glands were implanted at three-day intervals into the peritoneal cavity of immature *Fundulus*. At the end of four weeks all twenty-four experimental animals of both sexes showed a high degree of gonadal stimulation.

This was particularly notable in the males where large numbers of mature spermatozoa and pigmentation characteristic of the adult during the breeding season were produced. The success of this method of implantation, which continuously supplied the young individual with fresh gland material, indicates that the failure in most cases of the method employing intra-ocular implants was due to a failure of the implanted gland to establish itself successfully so that it provided an inadequate supply of gonad-stimulating substances to the host.

The precocious development of the gonads produced by implanting the adult pituitary gland strongly suggests that the normal development of the mature condition of the gonad in *Fundulus* is controlled by the pituitary gland.

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THE TRANSPORT OF OXYGEN BY THE BLOOD OF FRESHWATER FISH

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INTRODUCTION

The stresses imposed by low oxygen pressures upon the ability of fish to oxygenate their blood and by low temperatures upon their ability to utilize oxygen in the blood were first appreciated by Krogh and Leitch (1919). In their study of four species of freshwater fish and two marine species they noted the apparent relationship between the pressure of oxygen required to oxygenate the blood and the pressure of oxygen in the external environment in which the species live. They stated that the difference in the properties of the blood (between fish living in poorly oxygenated water and well oxygenated water) was of such a magnitude as to suggest species differences. They also noted that carbon dioxide had a greater effect on the blood of fish than on mammalian blood in reducing the affinity of the hemoglobin for oxygen. This effect, known as the "Bohr effect" is thought to be an advantage in the transport of oxygen in that it should raise the pressure at which oxygen is discharged from the blood and thus should facilitate diffusion of oxygen to the tissues. The latter property, namely the enlarged Bohr effect, was considered by Krogh and Leitch as an adaptation to low environmental temperature, while the possession of a higher affinity to oxygen was regarded as an adaptation to low environmental oxygen tension. They suggested that these adaptations were made possible by special conditions within the erythrocyte affecting the hemoglobin.

Root (1931), in his careful examination of the blood of several marine teleosts, found that carbon dioxide reduced the maximal amount of oxygen combined, even in the presence of as high a pressure of oxygen as 150 mm. Hg. He found too that the blood of each species was very sensitive to carbon dioxide compared with mammalian blood, but his data do not indicate that the affinity of the hemoglobin varies significantly from species to species. Willmer (1934), in a study of the respiration of tropical freshwater fish, noted amongst other features that the blood varied in sensitivity toward carbon dioxide. He observed a relation between the environment of the fish and the sensi-

tivity of the blood towards carbon dioxide, the allegedly active river fish having the most sensitive blood and the fish of the swamp possessing the least sensitive blood. Powers *et al.* (1932, 1938) have presented some data on oxygen dissociation of freshwater fish blood and on the relation of environmental gases to blood gases. Black and Irving (1938) showed that the ability of carbon dioxide to alter the affinity of blood of certain fish depends largely upon the intact corpuscle.

The present paper deals with certain characteristics of the blood of four species of freshwater fish, which determine the capacity of the blood of the fish for the transport of oxygen. This has been accomplished by the construction of oxygen dissociation curves for whole blood at various pressures of carbon dioxide and at one temperature (15° C.). Comparison of the properties of the blood can only be made at the same temperature, and the temperature chosen was one at which all the species could survive with apparent ease. This allows the correlation of the properties of the blood with the environmental characteristics of the fish, but restricts deductions as to the natural functioning of the blood of each species in its characteristic environment whatever that may be.

The results obtained show specific differences in the affinity of the hemoglobin and, moreover, specific differences in the sensitivity of the whole blood to carbon dioxide, an effect which has been shown (Black and Irving, 1938) to depend in a large degree upon the intact erythrocyte. In addition, data are presented which show that blood of high oxygen affinity has a low sensitivity to carbon dioxide, and blood of low oxygen affinity possesses a high sensitivity to carbon dioxide.

Materials and Methods

In this study four species of freshwater fish were used. These represented two superorders, Ganoidei and Teleosti of the subclass Actinopteri. The ganoid fish investigated was the bowfin, *Amia calva* Linnaeus. The teleost fish employed were the common sucker, *Catostomus commersonnii* (Lacépède), the carp, *Cyprinus carpio* Linnaeus and the catfish, *Ameiurus nebulosus* (Le Sueur).

The bowfin were obtained from the Great Lakes while the common suckers and carp were caught in Ontario lakes (Scugog and Erie). The catfish were provided by the Torresdale fish hatchery (Delaware river).

Blood was drawn from the heart by means of a Luer syringe. In the case of the three larger species, blood from a single fish was used for a number of curves. Dry heparin was added to prevent clotting. In no instance was any substance employed to prevent the normal respiration of the cells.

Immediately after the blood was drawn it was transferred to a tonometer of the type described by Irving and Black (1937). The gas mixtures were made up manometrically and the blood-gas mixture equilibrated in a water bath for not less than 20 minutes at 15° C. Emphasis should be placed on this fact, that only the drawn blood was equilibrated at 15° C. and not the fish. The common suckers and carp were taken from water, the temperature of which varied from 5° to 15° C.

The size of the sample of blood used for each analysis was 0.5 ml. Oxygen and CO_2 were determined by means of the "combined method" of Van Slyke and Neill. The reaction mixture was shaken in the manometric Van Slyke apparatus for at least 6 minutes, for oxygen is

TABLE I

The oxygen capacity and red cell volume of the blood of four freshwater fish

Species	Number of fish	Oxygen capacity vol. % HbO_2	Red cell volume %	Oxygen capacity 100 ml. cells vol. % HbO_2
Catfish, *Ameiurus nebulosus*	15			
Mean		13.3	32.2	41
Range		8.4–17.4	20.5–39.7	34–46
Carp, *Cyprinus carpio*	16			
Mean		12.5	33.1	37
Range		6.4–22.9	16.4–61.0	34–44
Bowfin, *Amia calva*	5			
Mean		11.8	27.1	44
Range		8.5–13.9	25.6–30.7	42–45
Common sucker, *Catostomus commersonnii*	39			
Mean		10.6	32.5	33
Range		5.9–14.6	20.4–48.5	21–43

often only slowly extracted from fish blood. The agreement between two consecutive determinations of oxygen capacity was within 0.3 volumes per cent. The gas phase was analyzed in a Haldane apparatus.

The factors used in the calculations were taken from tables given by Peters and Van Slyke (1932). In all cases the amount of physically dissolved oxygen was calculated using a solubility coefficient of 0.036 and subtracted so that the values given for oxygen represent the quantities combined with hemoglobin.

EXPERIMENTAL RESULTS

Oxygen Capacities and Red Cell Volumes

The upper limit of the quantity of oxygen which a given volume of blood can transport is determined by the oxygen capacity of the blood.

By oxygen capacity is meant the amount of oxygen (expressed as volumes per cent) combined with hemoglobin of whole blood when the pressure of oxygen is 153 mm. (that of air) and when the pressure of CO_2, as shown by analysis of the gas phase, is less than 2 mm. Hg.

Individuals of all four species showed considerable variation in the oxygen capacity. The differences in the mean values for the four species as compared with the individual variations are small and hence the differences in the mean values are of no apparent significance. In comparing the oxygen capacities listed in Table I with the oxygen capacities of various animals compiled by Redfield (1933), the average value for the catfish, carp, bowfin and common sucker is higher than that for most marine fish but is well below the average for mammals.

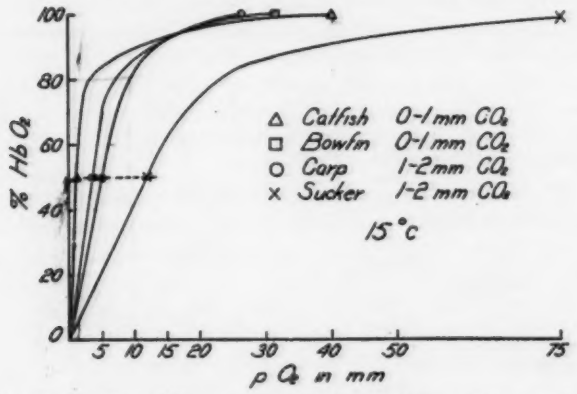

FIG. 1. Oxygen dissociation curves for the blood of the catfish, carp, bowfin and common sucker at pCO_2 0–2 mm.

It is of some interest to note that the mean oxygen capacity for the bowfin, a ganoid, is about equal to the average of the bony fishes while it is twice that found for elasmobranchs by Dill, Edwards and Florkin (1932) and Ferguson, Horvath and Pappenheimer (1938).

Table I also gives the relation of oxygen capacity to red cell volume. Since red cell volume in any one sample of blood varied as a result of conditions not as yet completely investigated, little significance is attached to the values given.

The Combination of Oxygen with Blood

Oxygen Dissociation Curves in the Absence of CO_2.—Because of the metabolism of the nucleated erythrocytes in fish blood it is difficult to obtain oxygen dissociation data in the complete absence of CO_2. In-

asmuch as the effect of pCO_2 2 mm. could not be discriminated from the effect of an indetectable pressure of CO_2 for the blood of the most sensitive fish, the oxygen dissociation curves for all the species at pCO_2 0–2 mm. are considered to be in the virtual absence of CO_2.

Oxygen dissociation curves for the blood of the four species are shown in Fig. 1. The data for the carp are taken from work published previously by Black and Irving (1937); the full data for the other three species are plotted in Fig. 2 (common sucker), Fig. 3 (catfish) and Fig. 4 (bowfin).

The curves for the four species seem to constitute one family of curves rising from the origin without evidence of an inflection which may, however, be present at higher temperatures.

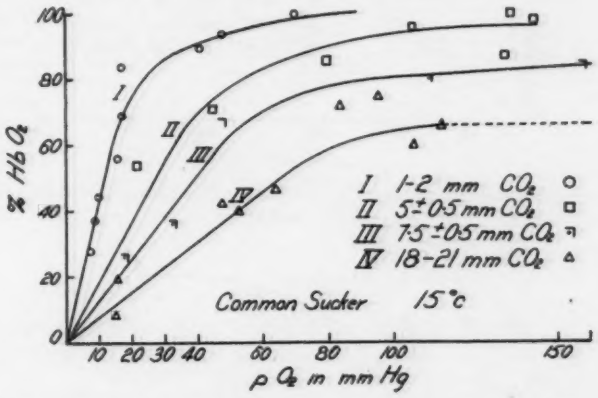

FIG. 2. Oxygen dissociation curves for the blood of the common sucker at various pressures of CO_2. (N. B. Change in scale.)

The pressure of oxygen required to half saturate the blood is often taken as a characteristic of the dissociation curve. Krogh and Leitch (1919) called this the tension of unloading (t_u). This point does not necessarily represent the true conditions for unloading of fish blood so that the pressure at half saturation is used in this paper merely to represent the mid-point of the functional range of the hemoglobin. Further, the pressure of half saturation may be used as a single figure to represent the oxygen affinity of the blood, when the blood is equilibrated in the virtual absence of CO_2. These pressures are given in Table II and reflect the fact that the curves for the catfish, bowfin and carp rise more steeply than that for the common sucker. They also indicate that, under these conditions of CO_2 and temperature, the

oxygen of the blood of the common sucker would be available to the tissues at higher pressures than in the other three fish.

Krogh and Leitch (1919) have also introduced the term "tension of loading" (t_l) to designate the pressure for 95 per cent saturation of

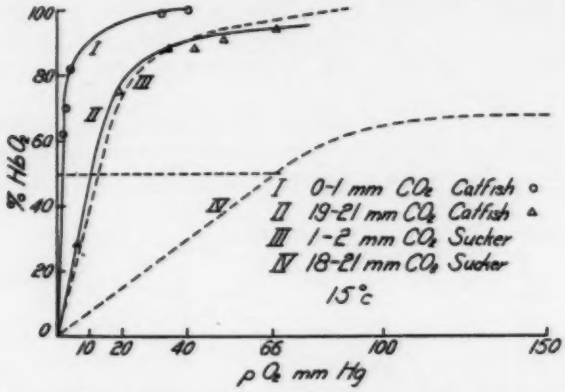

FIG. 3. Oxygen dissociation curves for the blood of the catfish and common sucker at pCO_2 0–2 and 20 mm.

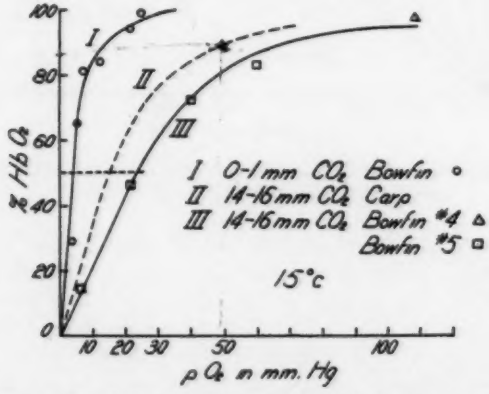

FIG. 4. Oxygen dissociation curves for the blood of the carp at pCO_2 15 mm. and for the blood of the bowfin at 1 and 15 mm. CO_2.

the blood. This is a common degree of saturation for mammalian arterial blood but little satisfactory data exist to show the conditions of the blood coming from the gills of the fish. The loading tensions might also be used to designate the affinity of the hemoglobin for

oxygen and in fact they do in this series correspond qualitatively to the tension of unloading. They are not used here because they cannot be determined with any satisfactory degree of precision owing to the rapidly changing slope of the curves in this region.

The oxygen dissociation curves for blood *equilibrated in the absence of* CO_2 are practically identical for the carp and the bowfin and they

TABLE II

Pressures of oxygen required for half saturation and loading of fish blood at various tensions of carbon dioxide and equilibrated at 15° C.

Species	pCO_2	pO_2 at 50% HbO_2	pO_2 at 95% HbO_2
	mm. Hg	*mm. Hg*	*mm. Hg*
Catfish			
Sample 5 (single)	0–1	1.4	18
Sample 9 (4 pooled)	19–21	10	75
Sample 5 (single)	39	7.5	65
Sample 7 (2 pooled)	41	9	65
Sample 8 (2 pooled)	38	14	85
Carp			
(mean values of plotted data, Black and Irving, 1937)	1–2	5	17
	9–11	8	35
	14–16	14	70
	18–20	20	—
Bowfin			
Sample 4 (single)	0–1	4	19
Sample 5 (single)	12	11	
Sample 5 (single)	15	23	110
Common sucker			
(mean values of plotted data, Fig. 2)	1–2	12	53
	5	27	105
	7.5	40	—
	12*	52	—
	18–21	66	—

* Single point.

stand midway between the widely separated curves for the catfish and the common sucker (Fig. 1). From these data it may be said that the affinity for oxygen of the hemoglobin as it exists in the blood differs significantly in certain species of fish, a generalization first stated by Krogh and Leitch (1919). The marked differences found for the species mentioned above are in contrast to the similarity of the oxygen affinity of the blood of the five marine fish studied by Root (1931) and Green and Root (1933).

The Effect of CO_2 upon the Affinity for Oxygen of Whole Blood Studied

Common Sucker.—It is evident that CO_2 not only decreased the affinity for oxygen in the sense of increasing the pressure of oxygen required for half saturation, but also lowered the maximal amount of oxygen combined, *i.e.* at 150 mm. oxygen, as found by Root for certain marine teleosts. It has been demonstrated on carp and rainbow trout blood by Ferguson and Black (1940) that in the presence of CO_2 even a pressure of 700 mm. oxygen failed to restore the amount of oxygen combined with the hemoglobin to its former value.

It is apparent from Fig. 2 that increasing the pCO_2 from 2 to 20 mm. increases the oxygen pressure at half saturation by about 50 mm. The extreme individual variations found in seven fish give this mean figure a maximal spread of ± 6 mm. of oxygen. The magnitude of the sensitivity to CO_2 of the blood of the common sucker has been further confirmed on blood from nine other fish exposed to tensions of CO_2 of $5 \pm .5$ and $7.5 \pm .5$ mm.

Catfish.—The blood of the catfish showed the least sensitivity to CO_2 both as to decrease of maximal oxygen combined at high oxygen pressures and as to increased pressures of oxygen required at half saturation. Figure 3 contrasts the effect of CO_2 on catfish blood with mean curves of the blood of the common sucker.

The curve plotted for the blood of the catfish at pCO_2 20 mm. represents the sample most sensitive to CO_2 but it shows an increase of only 8 mm. in the pressure of oxygen at half saturation and is in sharp contrast to the mean figure of 50 mm. for the common sucker. The difference between these species in sensitivity to CO_2 is far beyond the range of individual variation.

Carp and Bowfin.—Data for carp and bowfin are plotted in Fig. 4. The CO_2 sensitivities of the blood of these two species do not appear to differ greatly. The sensitivities are between those of the common sucker and the catfish but much closer to the latter. Very extensive studies would be necessary to establish a significant species difference between the carp and the bowfin though it may well exist.

Discussion

Von Ledebur (1939) cites Hall (1929) and Root (1931) and concludes that in certain instances, especially among marine fish, there seems to be a parallelism between the oxygen affinity of the hemoglobin and the activity of the species. However, Hall (1929) stated that in the three species studied the correlation was with the *amount* of hemoglobin per unit volume of blood, not with oxygen *affinity* of the hemoglobin. Root (1931) stated that "sluggish fish have bloods of low oxygen

capacity and the active of high capacity." Our data on oxygen capacities allow no deductions on this point since significant species differences were not found.

The differences in oxygen affinity are quite striking. An examination of Fig. 1 shows that the blood of the carp could be oxygenated to the extent of 90 per cent at a pressure of oxygen of 12 mm., at which pressure the blood of the common sucker is only half saturated. Again the remarkable blood of the catfish could serve at an environmental oxygen tension quite unsuited to the blood of the other species, for at pO_2 3 mm. its blood would be 80 per cent saturated while the blood of the carp and common sucker would only be 35 and 12 per cent oxygenated respectively.

Krogh and Leitch (1919) suggested a relation between the affinity of the blood for oxygen with the ability of the species to survive in environments low in oxygen. That this is an important relation is supported by the work of Fry and Black (1938), who have shown that the catfish, *A. nebulosus*, was able to remove oxygen at a lower tension than any of the other fish among the large series of fishes studied; as Wells (1913) found to be the case for a related species of catfish, *A. melas*.

The significance of the species difference in CO_2 sensitivity of the blood in relation to the ability of the blood to transport oxygen may be seen by considering the following hypothetical situations. If blood be fully oxygenated at the gills in the absence of CO_2 and transported to the tissues where metabolism proceeds until the pressure of both CO_2 and oxygen is 20 mm., then 25 per cent of the combined oxygen could be discharged from the blood of the catfish, 50 per cent from that of the carp and 85 per cent from the blood of the common sucker. Or viewed in another manner, the blood of the common sucker can be loaded (95 per cent HbO_2) at a pressure of 53 mm. Were this blood now exposed to the tissues where a pressure of 20 mm. CO_2 obtained, then 55 per cent (from 95 to 40 volumes per cent) of the combined oxygen could be utilized without any loss in the pressure of oxygen first required for loading. Under similar circumstances only about 15 per cent (from 95 to 80 volumes per cent) of the combined oxygen could be discharged by the blood of the catfish at its loading pressure of about 18 mm.

In terms of pressure of oxygen produced (at constant oxygenation) the specific differences in the ability of CO_2 to affect the equilibrium between oxygen and whole blood are no less interesting. If blood first oxygenated at the gills in the absence of CO_2 be transferred to the tissues where a tension of 20 mm. CO_2 exists, then when one-third

of the combined oxygen is used, the pressures of oxygen available to the tissues will be of the order of 15 mm. in the catfish, 30 mm. for the carp and 115 mm. for the common sucker.

It is an accepted concept of mammalian physiology that the Bohr effect contributes significantly to the efficiency of oxygen transport and might under certain circumstances be an essential factor in transportation of oxygen. Consequently it was thought that the wide differences in CO_2 sensitivity might reflect differences in habitual activity or capacity for maximal activity. Unfortunately, little data are available on the activity of fishes, habitual or maximal, in their natural environment or on metabolism, basal or maximal.

If CO_2 sensitivity of the blood is related to activity one would espect that the maximal activity of the catfish would be of a very low order. However, the maximal oxygen consumption of actively swimming catfish was found to be greater than the maximal oxygen utilization of the perch and common sunfish (Black, Fry and Scott, 1939). The blood of the perch is much more sensitive to CO_2 than the blood of the catfish (Irving, Black and Safford, 1939). It would seem then that no correlation between the CO_2 sensitivity of the blood and the rate of oxygen uptake of actively swimming fish is apparent. From this the inference is made that while the enlarged Bohr effect must be of importance to the efficiency of oxygen transport, each of the other factors such as circulatory efficiency, environmental temperature, the tension at which tissues can utilize oxygen, etc. is so important that the existence of a correlation between the activity of the fish and the single effect of CO_2 upon oxygen affinity of the hemoglobin may be masked. For example, J. S. Hart (1940) in his estimation of cardiac output for the catfish, carp, bowfin and common sucker found that the stroke volume of the catfish greatly exceeded the others while the heart of the common sucker had the smallest output per beat.

The possibility that the CO_2 effect upon the oxygen affinity may function as an oxygen pump is of the utmost interest in connection with the swimbladder. In their efforts to determine whether the mammalian lung secreted oxygen, Bohr and Haldane were greatly concerned with the mechanism for the deposition of gases in the swimbladder. An inspection of the oxygen dissociation curves of the blood of the common sucker at various pressures of CO_2 shows that the pressure of oxygen could be greatly increased by small pressures of CO_2. These data extend the quantitative basis for the theory as put forth by Haldane (1922), Hall (1924) and Jacobs (1930). Of particular significance is the experimental demonstration (mentioned in results) that CO_2 will dissociate oxygen from hemoglobin at pressures up to 700 mm. Hg.

While the increased sensitivity to CO_2 should enhance the use of the blood in transporting oxygen and in making available a higher pressure of oxygen at unloading, this very sensitivity should hinder oxygenation at the gills. Fry and Black (1938) tested this implication and found that the common sucker with its CO_2-sensitive blood was unable to remove oxygen from water containing CO_2 at tensions which did not hinder the respiration of catfish. Catfish proved to be the most resistant to the presence of CO_2 of all species studied, and it is a well-known fact that catfish are often found in swamps of acid water.

Although there is little evidence of significant differences in oxygen capacity, the experiments suggest strongly that the differences (a) in oxygen affinity of the blood and (b) sensitivity of the oxygen affinity to CO_2, are important in natural respiration. Krogh and Leitch suggested that species variations might be considered as adaptations to environmental conditions, specifying particularly the degree of saturation of the water with oxygen. The experiments reported show that in the catfish a high oxygen affinity is associated with low sensitivity to CO_2 while in the common sucker a low oxygen affinity is associated with a high CO_2 sensitivity. If specific differences in oxygen affinity constitute a basis for adaptation, then differences in CO_2 sensitivity of the blood must be considered as part of the adaptation of the blood. Krogh and Leitch recognized the importance of the CO_2 effect but did not observe that it differed greatly among the species.

The possession of blood characterized by a high affinity for oxygen would be expected to allow utilization of oxygen from water in which it was dissolved at a low tension. Such a hemoglobin, however, would impose the disadvantage that this oxygen would only be available to the tissues at a low pressure. This disadvantage might be offset by a high sensitivity to CO_2 whereby the CO_2 in the tissues would release the oxygen at a higher pressure. However, these experiments show that the catfish which has the highest oxygen affinity has no great CO_2 effect to offset it but indeed has the smallest CO_2 effect of the fish studied.

One other factor which might offset the apparent disadvantage of high oxygen affinity would be a higher environmental temperature, for higher temperature, like CO_2, also tends to lower oxygen affinity or raise the pressure of unloading (t_u). It appears that the catfish in the summer frequents the shallower warmer water (Fry, 1939) and it is now apparent how the characteristics of the blood of the catfish should be more suitable in the warmer than in cooler strata of water.

The common sucker is found in deeper water in summer (Fry, 1939) where the possession of lower oxygen affinity and higher CO_2 sensitivity

offset the tendency of the lower temperature to decrease the pressure of unloading (t_u).

Experiments of Irving, Black and Safford (1940) (done at 15° C.) have shown that three species of trout (which are typically found in cold water), possess blood of even lower affinity for oxygen and greater sensitivity to CO_2 than that of the common sucker.

The low sensitivity to CO_2 and the high affinity for oxygen of the blood of the catfish are properties which could best be utilized for the transport of oxygen at higher rather than lower environmental temperatures. Conversely a low oxygen affinity and a high CO_2 sensitivity of the blood (such as characterize that of the common sucker or the trout) are properties which would tend to offset the lowered temperature effect in the transport of oxygen by the blood. The separate adaptive features of lowered oxygen affinity and enlarged Bohr effect of fish blood in an environment of low temperature were set forth by Krogh and Leitch (1919). However, their data did not show that high affinity for oxygen is in general possessed by blood of low CO_2 sensitivity and that blood of low affinity for oxygen has a large CO_2 effect. Moreover, their data did not show that species differences existed in the sensitivity of the blood to CO_2. That gradations in the CO_2 effect may be of importance in the economy of fish inhabiting different thermal strata of freshwater lakes has been discussed very clearly by Fry (1939).

Krogh and Leitch suggested that the specific characteristics of the hemoglobin in the different bloods must be due to specific conditions in the different erythrocytes. This view was based on the belief common at that time that the hemoglobin was identical in all species. It is now known that considerable differences in the properties can be found due to differences in the globin. It remains to be shown to what extent the specific characteristics of these bloods are due to different hemoglobins and to what extent due to special conditions in the erythrocytes. Hall and McCutcheon (1938) have made an extensive examination of the differences in the hemoglobins of marine fish. It seems important to point out that these differences in hemoglobins do not always explain the sum total of the differences between whole bloods as regards oxygen transport, for Black and Irving (1938) have shown for the blood of the carp, bowfin and common sucker that when hemoglobin is released by hemolysis the effects of CO_2 on oxygen affinity become so strikingly different that it is at present impossible to predict the properties of the hemoglobin within the red cell from its observed properties in solution. The explanation given by Krogh and Leitch for the basis of adaptation in the blood of fish

may then be extended, for the specificity of the oxygen affinity of the hemoglobin and the sensitivity of the hemoglobin to CO_2 as it exists in fish blood are undoubtedly a combination of the two factors, specificity of the hemoglobin moiety and the specific influence of the intact corpuscle.

Summary

1. Oxygen capacities have been determined on the blood of four freshwater fish (catfish, carp, bowfin and common sucker). Individual variations are larger than average interspecific differences.

2. Significant differences between species were found for the affinity of the hemoglobin in whole blood for oxygen in the absence of CO_2 and at 15° C. in these freshwater fish as contrasted with the rather uniform oxygen affinity of most marine fish studied so far.

3. Significant differences between species were found for the effect of CO_2 on the affinity of the hemoglobin in whole blood for oxygen. For example, at 15° C. an increase in pCO_2 from 2 to 20 mm. causes an increase in oxygen pressure at half saturation of 8 mm. for the catfish and of 50 mm. for the common sucker.

4. Blood with high oxygen affinity has a low sensitivity to CO_2 (catfish). Blood with low affinity for oxygen has a high CO_2 sensitivity (common sucker).

5. It is suggested that since blood characterized by high oxygen affinity lacks a large CO_2 effect which could offset the disadvantage imposed at deoxygenation, the blood would function best in a high environmental temperature.

6. The greater sensitivity to CO_2 of fish blood as compared with mammalian blood was regarded by Krogh and Leitch as an adaptation to lower temperature. Evidence can now be presented to show that freshwater fish of higher CO_2 sensitivities are found in the colder habitats.

7. The very striking effects of CO_2 show that high pressures of oxygen might be developed within the fish by the presence of relatively low pressures of CO_2. The old suggestion that the Bohr effect may be responsible for the high percentage of oxygen in the swimbladder of many fish is thus given additional quantitative support.

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